

GENE EXPRESSION PROFILING OF EGFR POSITIVE CANCERBackground of the Invention

[0001] This application claims priority under 35 U.S.C. § 119(e) to provisional application Serial No. 60/427,090 filed on November 15, 2002, the entire disclosure of which is hereby expressly incorporated by reference.

Field of the Invention

[0002] The present invention concerns gene expression profiling of tissue samples obtained from EGFR-positive cancer. More specifically, the invention provides diagnostic, prognostic and predictive methods based on the molecular characterization of gene expression in paraffin-embedded, fixed tissue samples of EGFR-expressing cancer, which allow a physician to predict whether a patient is likely to respond well to treatment with an EGFR inhibitor. In addition, the present invention provides treatment methods based on such findings.

Description of the Related Art

[0003] Oncologists have a number of treatment options available to them, including different combinations of chemotherapeutic drugs that are characterized as "standard of care," and a number of drugs that do not carry a label claim for particular cancer, but for which there is evidence of efficacy in that cancer. Best likelihood of good treatment outcome requires that patients be assigned to optimal available cancer treatment, and that this assignment be made as quickly as possible following diagnosis.

[0004] Currently, diagnostic tests used in clinical practice are single analyte, and therefore do not capture the potential value of knowing relationships between dozens of different markers. Moreover, diagnostic tests are frequently not quantitative, relying on immunohistochemistry. This method often yields different results in different laboratories, in part because the reagents are not standardized, and in part because the interpretations are subjective and cannot be easily quantified. RNA-based tests have not often been used because of the problem of RNA degradation over time and the fact that it is difficult to obtain fresh tissue samples from patients for analysis. Fixed paraffin-embedded tissue is more readily available and methods have been established to detect RNA in fixed tissue. However, these methods typically do not allow for the study of large numbers of genes (DNA or RNA)

from small amounts of material. Thus, traditionally fixed tissue has been rarely used other than for immunohistochemistry detection of proteins.

[0005] Recently, several groups have published studies concerning the classification of various cancer types by microarray gene expression analysis (see, e.g. Golub *et al.*, *Science* 286:531-537 (1999); Bhattacharjee *et al.*, *Proc. Natl. Acad. Sci. USA* 98:13790-13795 (2001); Chen-Hsiang *et al.*, *Bioinformatics* 17 (Suppl. 1):S316-S322 (2001); Ramaswamy *et al.*, *Proc. Natl. Acad. Sci. USA* 98:15149-15154 (2001)). Certain classifications of human breast cancers based on gene expression patterns have also been reported (Martin *et al.*, *Cancer Res.* 60:2232-2238 (2000); West *et al.*, *Proc. Natl. Acad. Sci. USA* 98:11462-11467 (2001); Sorlie *et al.*, *Proc. Natl. Acad. Sci. USA* 98:10869-10874 (2001); Yan *et al.*, *Cancer Res.* 61:8375-8380 (2001)). However, these studies mostly focus on improving and refining the already established classification of various types of cancer, including breast cancer, and generally do not link the findings to treatment strategies in order to improve the clinical outcome of cancer therapy.

[0006] Although modern molecular biology and biochemistry have revealed more than 100 genes whose activities influence the behavior of tumor cells, state of their differentiation, and their sensitivity or resistance to certain therapeutic drugs, with a few exceptions, the status of these genes has not been exploited for the purpose of routinely making clinical decisions about drug treatments. One notable exception is the use of estrogen receptor (ER) protein expression in breast carcinomas to select patients to treatment with anti-estrogen drugs, such as tamoxifen. Another exceptional example is the use of ErbB2 (Her2) protein expression in breast carcinomas to select patients with the Her2 antagonist drug Herceptin® (Genentech, Inc., South San Francisco, CA).

[0007] Despite recent advances, the challenge of cancer treatment remains to target specific treatment regimens to pathogenically distinct tumor types, and ultimately personalize tumor treatment in order to optimize outcome. Hence, a need exists for tests that simultaneously provide predictive information about patient responses to the variety of treatment options.

Summary of the Invention

[0008] The present invention is based on findings of Phase II clinical studies of gene expression in tissue samples obtained from EGFR-expressing head and neck cancer or colon cancer of human patients who responded well or did not respond to (showed resistance to) treatment with EGFR inhibitors.

[0009] Based upon such findings, in one aspect the present invention concerns a method for predicting the likelihood that a patient diagnosed with an EGFR-expressing cancer will respond to treatment with an EGFR inhibitor, comprising determining the expression level of one or more prognostic RNA transcripts or their products in a sample comprising EGFR-expressing cancer cells obtained from the patient, wherein the prognostic transcript is the transcript of one or more genes selected from the group consisting of: Bak; Bclx; BRAF; BRK; Cad17; CCND3; CD105; CD44s; CD82; CD9; CGA;; CTSL; EGFRd27; ErbB3; EREG; GPC3; GUS; HGF; ID1; IGFBP3; ITGB3; ITGB3; p27; P53; PTPD1; RB1; RPLPO; STK15; SURV; TERC; TGFB2; TIMP2; TITF1; XIAP; YB-1; A-Catenin; AKT1; AKT2; APC; Bax; B-Catenin; BTC; CA9; CCNA2; CCNE1; CCNE2; CD134; CD44E; CD44v3; CD44v6; CD68; CDC25B; CEACAM6; Chk2; cMet; COX2; cripto; DCR3; DIABLO; DPYD; DR5; EDN1 endothelin; EGFR; EIF4E; ERBB4; ERK1; fas; FRP1; GRO1; HB-EGF; HER2; IGF1R; IRS1; ITGA3; KRT17; LAMC2; MTA1; NMYC; P14ARF; PAI1; PDGFA; PDGFB; PGK1; PLAUR; PPARG; RANBP2; RASSF1; RIZ1; SPRY2; Src; TFRC; TP53BP1;UPA; and VEGFC, wherein (a) the patient is unlikely to benefit from treatment with an EGFR inhibitor if the normalized levels of any of the following genes A-Catenin; AKT1; AKT2; APC; Bax; B-Catenin; BTC; CA9; CCNA2; CCNE1; CCNE2; CD134; CD44E; CD44v3; CD44v6; CD68; CDC25B; CEACAM6; Chk2; cMet; COX2; cripto; DCR3; DIABLO; DPYD; DR5; EDN1 endothelin; EGFR; EIF4E; ERBB4; ERK1; fas; FRP1; GRO1; HB-EGF; HER2; IGF1R; IRS1; ITGA3; KRT17; LAMC2; MTA1; NMYC; P14ARF; PAI1; PDGFA; PDGFB; PGK1; PLAUR; PPARG; RANBP2; RASSF1; RIZ1; SPRY2; Src; TFRC; TP53BP1; upa; VEGFC, or their products are elevated above defined expression thresholds, and (b) the patient is likely to benefit from treatment with an EGFR inhibitor if the normalized levels of any of the following genes Bak; Bclx; BRAF; BRK; Cad17; CCND3; CD105; CD44s; CD82; CD9; CGA;; CTSL; EGFRd27; ErbB3; EREG; GPC3; GUS; HGF; ID1; IGFBP3; ITGB3; ITGB3; p27; P53; PTPD1; RB1;

RPLPO; STK15; SURV; TERC; TGFB2; TIMP2; TITF1; XIAP; and YB-1, or their products are elevated above defined expression thresholds.

[0010] In another aspect, the present invention concerns a prognostic method comprising

(a) subjecting a sample comprising EGFR-expressing cancer cells obtained from a patient to quantitative analysis of the expression level of at least one gene selected from the group consisting of CD44v3; CD44v6; DR5; GRO1; KRT17; and LAMC2 gene or their products, and

(b) identifying the patient as likely to show resistance to treatment with an EGFR-inhibitor if the expression levels of such gene or genes, or their products, are elevated above a defined threshold. In a particular embodiment, the gene is LAMC2.

[0011] In yet another aspect, the invention concerns a method for predicting the likelihood that a patient diagnosed with an EGFR-expressing head or neck cancer will respond to treatment with an EGFR inhibitor, comprising determining the expression level of one or more prognostic RNA transcripts or their products in a sample comprising EGFR-expressing cancer cells obtained from such patient, wherein the prognostic transcript is the transcript of one or more genes selected from the group consisting of: CD44s; CD82; CGA; CTSL; EGFRd27; IGFBP3; p27; P53; RB1; TIMP2; YB-1; A-Catenin; AKT1; AKT2; APC; Bax; B-Catenin; BTC; CCNA2; CCNE1; CCNE2; CD105; CD44v3; CD44v6; CD68; CEACAM6; Chk2; cMet; COX2; cripto; DCR3; DIABLO; DPYD; DR5; EDN1 endothelin; EGFR; EIF4E; ERBB4; ERK1; fas; FRP1; GRO1; HB-EGF; HER2; IGF1R; IRS1; ITGA3; KRT17; LAMC2; MTA1; NMYC; PAI1; PDGFA; PGK1; PTPD1; RANBP2; SPRY2; TP53BP1; and VEGFC, wherein (a) normalized expression of one or more of A-Catenin; AKT1; AKT2; APC; Bax; B-Catenin; BTC; CCNA2; CCNE1; CCNE2; CD105; CD44v3; CD44v6; CD68; CEACAM6; Chk2; cMet; COX2; cripto; DCR3; DIABLO; DPYD; DR5; EDN1 endothelin; EGFR; EIF4E; ERBB4; ERK1; fas; FRP1; GRO1; HB-EGF; HER2; IGF1R; IRS1; ITGA3; KRT17; LAMC2; MTA1; NMYC; PAI1; PDGFA; PGK1; PTPD1; RANBP2; SPRY2; TP53BP1; VEGFC, or the corresponding gene product, above determined expression thresholds indicates that the patient is likely to show resistance to treatment with an EGFR inhibitor, and (b) normalized expression of one or more of CD44s; CD82; CGA; CTSL; EGFRd27; IGFBP3; p27; P53; RB1; TIMP2; YB-1, or the corresponding gene

product, above defined expression thresholds indicates that the patient is likely to respond well to treatment with an EGFR inhibitor.

[0012] In a further aspect, the invention concerns a method for predicting the likelihood that a patient diagnosed with an EGFR-expressing colon cancer will respond to treatment with an EGFR inhibitor, comprising determining the expression level of one or more prognostic RNA transcripts or their products in a sample comprising EGFR-expressing cancer cells obtained from the patient, wherein the prognostic transcript is the transcript of one or more genes selected from the group consisting of Bak; Bclx; BRAF; BRK; Cad17; CCND3; CCNE1; CCNE2; CD105; CD9; COX2; DIABLO; ErbB3; EREG; FRP1; GPC3; GUS; HER2; HGF; ID1; ITGB3; PTPD1; RPLPO; STK15; SURV; TERC; TGFBR2; TITF1; XIAP; CA9; CD134; CD44E; CD44v3; CD44v6; CDC25B; CGA; DR5; GRO1; KRT17; LAMC2; P14ARF; PDGFB; PLAUR; PPARG; RASSF1; RIZ1; Src; TFRC; and UPA, wherein (a) elevated expression of one or more of CA9; CD134; CD44E; CD44v3; CD44v6; CDC25B; CGA; DR5; GRO1; KRT17; LAMC2; P14ARF; PDGFB; PLAUR; PPARG; RASSF1; RIZ1; Src; TFRC; and UPA, or the corresponding gene product, above defined expression thresholds indicates that the patient is likely to show resistance to treatment with an EGFR inhibitor, and normalized expression of one or more of Bak; Bclx; BRAF; BRK; Cad17; CCND3; CCNE1; CCNE2; CD105; CD9; COX2; DIABLO; ErbB3; EREG; FRP1; GPC3; GUS; HER2; HGF; ID1; ITGB3; PTPD1; RPLPO; STK15; SURV; TERC; TGFBR2; TITF1; XIAP, or the corresponding gene product, above certain expression thresholds indicates that the patient is likely to respond well to treatment with an EGFR inhibitor.

[0013] In another aspect, the invention concerns a method comprising treating a patient diagnosed with an EGFR-expressing cancer and determined to have elevated normalized levels of one or more of the RNA transcripts of Bak; Bclx; BRAF; BRK; Cad17; CCND3; CD105; CD44s; CD82; CD9; CGA; CTSL; EGFRd27; ErbB3; EREG; GPC3; GUS; HGF; ID1; IGFBP3; ITGB3; ITGB3; p27; P53; PTPD1; RB1; RPLPO; STK15; SURV; TERC; TGFBR2; TIMP2; TITF1; XIAP; YB-1; A-Catenin; AKT1; AKT2; APC; Bax; B-Catenin; BTC; CA9; CCNA2; CCNE1; CCNE2; CD134; CD44E; CD44v3; CD44v6; CD68; CDC25B; CEACAM6; Chk2; cMet; COX2; cripto; DCR3; DIABLO; DPYD; DR5; EDN1 endothelin; EGFR; EIF4E; ERBB4; ERK1; fas; FRP1; GRO1; HB-EGF; HER2; IGF1R; IRS1; ITGA3; KRT17; LAMC2; MTA1; NMYC; P14ARF; PAI1; PDGFA;

PDGFB; PGK1; PLAUR; PPARG; RANBP2; RASSF1; RIZ1; SPRY2; Src; TFRC; TP53BP1; UPA; and VEGFC genes, or the corresponding gene products in the cancer, with an effective amount of an EGFR-inhibitor, wherein elevated RNA transcript level is defined by a defined expression threshold.

[0014] In yet another aspect, the invention concerns a method comprising treating a patient diagnosed with an EGFR-expressing head or neck cancer and determined to have elevated normalized expression of one or more of the RNA transcripts of CD44s; CD82; CGA; CTSL; EGFRd27; IGFBP3; p27; P53; RB1; TIMP2; YB-1; A-Catenin; AKT1; AKT2; APC; Bax; B-Catenin; BTC; CCNA2; CCNE1; CCNE2; CD105; CD44v3; CD44v6; CD68; CEACAM6; Chk2; cMet; COX2; cripto; DCR3; DIABLO; DPYD; DR5; EDN1 endothelin; EGFR; EIF4E; ERBB4; ERK1; fas; FRP1; GRO1; HB-EGF; HER2; IGF1R; IRS1; ITGA3; KRT17; LAMC2; MTA1; NMYC; PAI1; PDGFA; PGK1; PTPD1; RANBP2; SPRY2; TP53BP1; VEGFC genes, or the corresponding gene products in said cancer, with an effective amount of an EGFR-inhibitor, wherein elevated normalized RNA transcript level is defined by a defined expression threshold.

[0015] In a further aspect, the invention concerns a method comprising treating a patient diagnosed with an EGFR-expressing colon cancer and determined to have elevated normalized expression of one or more of the RNA transcripts of Bak; Bclx; BRAF; BRK; Cad17; CCND3; CCNE1; CCNE2; CD105; CD9; COX2; DIABLO; ErbB3; EREG; FRP1; GPC3; GUS; HER2; HGF; ID1; ITGB3; PTPD1; RPLPO; STK15; SURV; TERC; TGFB2; TITF1; XIAP; CA9; CD134; CD44E; CD44v3; CD44v6; CDC25B; CGA; DR5; GRO1; KRT17; LAMC2; P14ARF; PDGFB; PLAUR; PPARG; RASSF1; RIZ1; Src; TFRC; UPA genes, or the corresponding gene products in such cancer, with an effective amount of an EGFR-inhibitor, wherein elevated normalized RNA transcript level is defined by a defined expression threshold.

[0016] The invention further concerns an array comprising (a) polynucleotides hybridizing to the following genes: Bak; Bclx; BRAF; BRK; Cad17; CCND3; CD105; CD44s; CD82; CD9; CGA;; CTSL; EGFRd27; ErbB3; EREG; GPC3; GUS; HGF; ID1; IGFBP3; ITGB3; ITGB3; p27; P53; PTPD1; RB1; RPLPO; STK15; SURV; TERC; TGFB2; TIMP2; TITF1; XIAP; YB-1; A-Catenin; AKT1; AKT2; APC; Bax; B-Catenin; BTC; CA9; CCNA2; CCNE1; CCNE2; CD134; CD44E; CD44v3; CD44v6; CD68;

CDC25B; CEACAM6; Chk2; cMet; COX2; cripto; DCR3; DIABLO; DPYD; DR5; EDN1 endothelin; EGFR; EIF4E; ERBB4; ERK1; fas; FRP1; GRO1; HB-EGF; HER2; IGF1R; IRS1; ITGA3; KRT17; LAMC2; MTA1; NMYC; P14ARF; PAI1; PDGFA; PDGFB; PGK1; PLAUR; PPARG; RANBP2; RASSF1; RIZ1; SPRY2; Src; TFRC; TP53BP1; UPA; VEGFC; or (b) an array comprising polynucleotides hybridizing to the following genes: CD44v3; CD44v6; DR5; GRO1; KRT17; and LAMC2, immobilized on a solid surface; or (c) an array comprising polynucleotides hybridizing to the following genes: CD44s; CD82; CGA; CTSL; EGFRd27; IGFBP3; p27; P53; RB1; TIMP2; YB-1; A-Catenin; AKT1; AKT2; APC; Bax; B-Catenin; BTC; CCNA2; CCNE1; CCNE2; CD105; CD44v3; CD44v6; CD68; CEACAM6; Chk2; cMet; COX2; cripto; DCR3; DIABLO; DPYD; DR5; EDN1 endothelin; EGFR; EIF4E; ERBB4; ERK1; fas; FRP1; GRO1; HB-EGF; HER2; IGF1R; IRS1; ITGA3; KRT17; LAMC2; MTA1; NMYC; PAI1; PDGFA; PGK1; PTPD1; RANBP2; SPRY2; TP53BP1; and VEGFC, immobilized on a solid surface, or (d) an array comprising polynucleotides hybridizing to the following genes: Bak; Bclx; BRAF; BRK; Cad17; CCND3; CCNE1; CCNE2; CD105; CD9; COX2; DIABLO; ErbB3; EREG; FRP1; GPC3; GUS; HER2; HGF; ID1; ITGB3; PTPD1; RPLPO; STK15; SURV; TERC; TGFB2; TITF1; XIAP; CA9; CD134; CD44E; CD44v3; CD44v6; CDC25B; CGA; DR5; GRO1; KRT17; LAMC2; P14ARF; PDGFB; PLAUR; PPARG; RASSF1; RIZ1; Src; TFRC; and UPA, immobilized on a solid surface.

[0017] In a further aspect, the invention concerns a method in which RNA is isolated from a fixed, paraffin-embedded tissue specimen by a procedure comprising:

- (a) incubating a section of the fixed, paraffin-embedded tissue specimen at a temperature of about 56 °C to 70 °C in a lysis buffer, in the presence of a protease, without prior dewaxing, to form a lysis solution;
- (b) cooling the lysis solution to a temperature where the wax solidifies; and
- (c) isolating the nucleic acid from the lysis solution.

[0018] In a different aspect, the invention concerns a kit comprising one or more of (1) extraction buffer/reagents and protocol; (2) reverse transcription buffer/reagents and protocol; and (3) qPCR buffer/reagents and protocol suitable for performing the gene expression analysis methods of the invention.

[0019] In a further aspect, the invention concerns a method for measuring levels of mRNA products of genes listed in Tables 5A and 5B by quantitative RT-PCR (qRT-PCR) reaction, by using an amplicon listed in Tables 5A and 5B and a corresponding primer-probe set listed in Tables 6A-6F.

Brief Description of the Drawings

[0020] Figure 1 is a chart illustrating the overall workflow of the process of the invention for measurement of gene expression. In the Figure, FPET stands for "fixed paraffin-embedded tissue," and "RT-PCR" stands for "reverse transcriptase-PCR." RNA concentration is determined by using the commercial RiboGreen™ RNA Quantitation Reagent and Protocol.

[0021] Figure 2 is a flow chart showing the steps of an RNA extraction method according to the invention alongside a flow chart of a representative commercial method.

Detailed Description of the Preferred Embodiment

A. Definitions

[0022] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton *et al.*, Dictionary of Microbiology and Molecular Biology 2nd ed., J. Wiley & Sons (New York, NY 1994), and March, Advanced Organic Chemistry Reactions, Mechanisms and Structure 4th ed., John Wiley & Sons (New York, NY 1992), provide one skilled in the art with a general guide to many of the terms used in the present application.

[0023] One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Indeed, the present invention is in no way limited to the methods and materials described. For purposes of the present invention, the following terms are defined below.

[0024] The term "microarray" refers to an ordered arrangement of hybridizable array elements, preferably polynucleotide probes, on a substrate.

[0025] The term "polynucleotide," when used in singular or plural, generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA

or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as defined herein include, without limitation, single- and double-stranded DNA, DNA including single- and double-stranded regions, single- and double-stranded RNA, and RNA including single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or include single- and double-stranded regions. In addition, the term "polynucleotide" as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. The term "polynucleotide" specifically includes cDNAs. The term includes DNAs (including cDNAs) and RNAs that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritiated bases, are included within the term "polynucleotides" as defined herein. In general, the term "polynucleotide" embraces all chemically, enzymatically and/or metabolically modified forms of unmodified polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells.

[0026] The term "oligonucleotide" refers to a relatively short polynucleotide, including, without limitation, single-stranded deoxyribonucleotides, single- or double-stranded ribonucleotides, RNA:DNA hybrids and double-stranded DNAs. Oligonucleotides, such as single-stranded DNA probe oligonucleotides, are often synthesized by chemical methods, for example using automated oligonucleotide synthesizers that are commercially available. However, oligonucleotides can be made by a variety of other methods, including *in vitro* recombinant DNA-mediated techniques and by expression of DNAs in cells and organisms.

[0027] The terms "differentially expressed gene," "differential gene expression" and their synonyms, which are used interchangeably, refer to a gene whose expression is activated to a higher or lower level in a subject suffering from a disease, specifically cancer, such as breast cancer, relative to its expression in a normal or control subject. The terms also include genes whose expression is activated to a higher or lower level at different stages of

the same disease. It is also understood that a differentially expressed gene may be either activated or inhibited at the nucleic acid level or protein level, or may be subject to alternative splicing to result in a different polypeptide product. Such differences may be evidenced by a change in mRNA levels, surface expression, secretion or other partitioning of a polypeptide, for example. Differential gene expression may include a comparison of expression between two or more genes or their gene products, or a comparison of the ratios of the expression between two or more genes or their gene products, or even a comparison of two differently processed products of the same gene, which differ between normal subjects and subjects suffering from a disease, specifically cancer, or between various stages of the same disease. Differential expression includes both quantitative, as well as qualitative, differences in the temporal or cellular expression pattern in a gene or its expression products among, for example, normal and diseased cells, or among cells which have undergone different disease events or disease stages. For the purpose of this invention, "differential gene expression" is considered to be present when there is at least an about two-fold, preferably at least about four-fold, more preferably at least about six-fold, most preferably at least about ten-fold difference between the expression of a given gene in normal and diseased subjects, or in various stages of disease development in a diseased subject.

[0028] The term "normalized" with regard to a gene transcript or a gene expression product refers to the level of the transcript or gene expression product relative to the mean levels of transcripts/products of a set of reference genes, wherein the reference genes are either selected based on their minimal variation across, patients, tissues or treatments ("housekeeping genes"), or the reference genes are the totality of tested genes. In the latter case, which is commonly referred to as "global normalization", it is important that the total number of tested genes be relatively large, preferably greater than 50. Specifically, the term 'normalized' with respect to an RNA transcript refers to the transcript level relative to the mean of transcript levels of a set of reference genes. More specifically, the mean level of an RNA transcript as measured by TaqMan® RT-PCR refers to the Ct value minus the mean Ct values of a set of reference gene transcripts.

[0029] The terms "expression threshold," and "defined expression threshold" are used interchangeably and refer to the level of a gene or gene product in question above which the gene or gene product serves as a predictive marker for patient response or resistance to a

drug, in the present case an EGFR inhibitor drug. The threshold is defined experimentally from clinical studies such as those described in examples 1 and 2, below. The expression threshold can be selected either for maximum sensitivity (for example, to detect all responders to a drug), or for maximum selectivity (for example to detect only responders to a drug), or for minimum error.

[0030] The phrase "gene amplification" refers to a process by which multiple copies of a gene or gene fragment are formed in a particular cell or cell line. The duplicated region (a stretch of amplified DNA) is often referred to as "amplicon." Usually, the amount of the messenger RNA (mRNA) produced, *i.e.*, the level of gene expression, also increases in the proportion of the number of copies made of the particular gene expressed.

[0031] The term "diagnosis" is used herein to refer to the identification of a molecular or pathological state, disease or condition, such as the identification of a molecular subtype of head and neck cancer, colon cancer, or other type of cancer. The term "prognosis" is used herein to refer to the prediction of the likelihood of cancer-attributable death or progression, including recurrence, metastatic spread, and drug resistance, of a neoplastic disease, such as breast cancer, or head and neck cancer. The term "prediction" is used herein to refer to the likelihood that a patient will respond either favorably or unfavorably to a drug or set of drugs, and also the extent of those responses, or that a patient will survive, following surgical removal of the primary tumor and/or chemotherapy for a certain period of time without cancer recurrence. The predictive methods of the present invention can be used clinically to make treatment decisions by choosing the most appropriate treatment modalities for any particular patient. The predictive methods of the present invention are valuable tools in predicting if a patient is likely to respond favorably to a treatment regimen, such as surgical intervention, chemotherapy with a given drug or drug combination, and/or radiation therapy, or whether long-term survival of the patient, following surgery and/or termination of chemotherapy or other treatment modalities is likely.

[0032] The term "long-term" survival is used herein to refer to survival for at least 5 years, more preferably for at least 8 years, most preferably for at least 10 years following surgery or other treatment.

[0033] The term "increased resistance" to a particular drug or treatment option, when used in accordance with the present invention, means decreased response to a standard dose of the drug or to a standard treatment protocol.

[0034] The term "decreased sensitivity" to a particular drug or treatment option, when used in accordance with the present invention, means decreased response to a standard dose of the drug or to a standard treatment protocol, where decreased response can be compensated for (at least partially) by increasing the dose of drug, or the intensity of treatment.

[0035] "Patient response" can be assessed using any endpoint indicating a benefit to the patient, including, without limitation, (1) inhibition, to some extent, of tumor growth, including slowing down and complete growth arrest; (2) reduction in the number of tumor cells; (3) reduction in tumor size; (4) inhibition (i.e., reduction, slowing down or complete stopping) of tumor cell infiltration into adjacent peripheral organs and/or tissues; (5) inhibition (i.e. reduction, slowing down or complete stopping) of metastasis; (6) enhancement of anti-tumor immune response, which may, but does not have to, result in the regression or rejection of the tumor; (7) relief, to some extent, of one or more symptoms associated with the tumor; (8) increase in the length of survival following treatment; and/or (9) decreased mortality at a given point of time following treatment.

[0036] The term "treatment" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented. In tumor (e.g., cancer) treatment, a therapeutic agent may directly decrease the pathology of tumor cells, or render the tumor cells more susceptible to treatment by other therapeutic agents, e.g., radiation and/or chemotherapy.

[0037] The term "tumor," as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

[0038] The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, breast cancer, colon cancer, lung cancer, prostate

cancer, hepatocellular cancer, gastric cancer, pancreatic cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, cancer of the urinary tract, thyroid cancer, renal cancer, carcinoma, melanoma, head and neck cancer, and brain cancer.

[0039] The "pathology" of cancer includes all phenomena that compromise the well-being of the patient. This includes, without limitation, abnormal or uncontrollable cell growth, metastasis, interference with the normal functioning of neighboring cells, release of cytokines or other secretory products at abnormal levels, suppression or aggravation of inflammatory or immunological response, neoplasia, premalignancy, malignancy, invasion of surrounding or distant tissues or organs, such as lymph nodes, etc.

[0040] The term "EGFR inhibitor" as used herein refers to a molecule having the ability to inhibit a biological function of a native epidermal growth factor receptor (EGFR). Accordingly, the term "inhibitor" is defined in the context of the biological role of EGFR. While preferred inhibitors herein specifically interact with (e.g. bind to) an EGFR, molecules that inhibit an EGFR biological activity by interacting with other members of the EGFR signal transduction pathway are also specifically included within this definition. A preferred EGFR biological activity inhibited by an EGFR inhibitor is associated with the development, growth, or spread of a tumor.

[0041] The term "housekeeping gene" refers to a group of genes that codes for proteins whose activities are essential for the maintenance of cell function. These genes are typically similarly expressed in all cell types. Housekeeping genes include, without limitation, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Cyp1, albumin, actins, e.g. β -actin, tubulins, cyclophilin, hypoxanthine phosphoribosyltransferase (HRPT), L32, 28S, and 18S.

B. Detailed Description

[0042] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, and biochemistry, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", 2nd edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology"

(Academic Press, Inc.); "Handbook of Experimental Immunology", 4th edition (D.M. Weir & C.C. Blackwell, eds., Blackwell Science Inc., 1987); "Gene Transfer Vectors for Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987); and "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994).

1. Gene Expression Profiling

[0043] In general, methods of gene expression profiling can be divided into two large groups: methods based on hybridization analysis of polynucleotides, and methods based on sequencing of polynucleotides. The most commonly used methods known in the art for the quantification of mRNA expression in a sample include northern blotting and *in situ* hybridization (Parker & Barnes, *Methods in Molecular Biology* 106:247-283 (1999)); RNase protection assays (Hod, *Biotechniques* 13:852-854 (1992)); and reverse transcription polymerase chain reaction (RT-PCR) (Weis *et al.*, *Trends in Genetics* 8:263-264 (1992)). Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. Representative methods for sequencing-based gene expression analysis include Serial Analysis of Gene Expression (SAGE), and gene expression analysis by massively parallel signature sequencing (MPSS).

2. Reverse Transcriptase PCR (RT-PCR)

[0044] Of the techniques listed above, the most sensitive and most flexible quantitative method is RT-PCR, which can be used to compare mRNA levels in different sample populations, in normal and tumor tissues, with or without drug treatment, to characterize patterns of gene expression, to discriminate between closely related mRNAs, and to analyze RNA structure.

[0045] The first step is the isolation of mRNA from a target sample. The starting material is typically total RNA isolated from human tumors or tumor cell lines, and corresponding normal tissues or cell lines, respectively. Thus RNA can be isolated from a variety of primary tumors, including breast, lung, colon, prostate, brain, liver, kidney, pancreas, spleen, thymus, testis, ovary, uterus, head and neck, etc., tumor, or tumor cell lines, with pooled DNA from healthy donors. If the source of mRNA is a primary tumor, mRNA can be extracted, for example, from frozen or archived paraffin-embedded and fixed (e.g. formalin-fixed) tissue samples.

[0046] General methods for mRNA extraction are well known in the art and are disclosed in standard textbooks of molecular biology, including Ausubel *et al.*, Current Protocols of Molecular Biology, John Wiley and Sons (1997). Methods for RNA extraction from paraffin embedded tissues are disclosed, for example, in Rupp and Locker, *Lab Invest.* 56:A67 (1987), and De Andrés *et al.*, *BioTechniques* 18:42044 (1995). In particular, RNA isolation can be performed using purification kit, buffer set and protease from commercial manufacturers, such as Qiagen, according to the manufacturer's instructions. For example, total RNA from cells in culture can be isolated using Qiagen RNeasy mini-columns. Other commercially available RNA isolation kits include MasterPure™ Complete DNA and RNA Purification Kit (EPICENTRE®, Madison, WI), and Paraffin Block RNA Isolation Kit (Ambion, Inc.). Total RNA from tissue samples can be isolated using RNA Stat-60 (Tel-Test). RNA prepared from tumor can be isolated, for example, by cesium chloride density gradient centrifugation.

[0047] As RNA cannot serve as a template for PCR, the first step in gene expression profiling by RT-PCR is the reverse transcription of the RNA template into cDNA, followed by its exponential amplification in a PCR reaction. The two most commonly used reverse transcriptases are avian myeloblastosis virus reverse transcriptase (AMV-RT) and

Moloney murine leukemia virus reverse transcriptase (MMLV-RT). The reverse transcription step is typically primed using specific primers, random hexamers, or oligo-dT primers, depending on the circumstances and the goal of expression profiling. For example, extracted RNA can be reverse-transcribed using a GeneAmp RNA PCR kit (Perkin Elmer, CA, USA), following the manufacturer's instructions. The derived cDNA can then be used as a template in the subsequent PCR reaction.

[0048] Although the PCR step can use a variety of thermostable DNA-dependent DNA polymerases, it typically employs the Taq DNA polymerase, which has a 5'-3' nuclease activity but lacks a 3'-5' proofreading endonuclease activity. Thus, TaqMan® PCR typically utilizes the 5'-nuclease activity of Taq or Tth polymerase to hydrolyze a hybridization probe bound to its target amplicon, but any enzyme with equivalent 5' nuclease activity can be used. Two oligonucleotide primers are used to generate an amplicon typical of a PCR reaction. A third oligonucleotide, or probe, is designed to detect nucleotide sequence located between the two PCR primers. The probe is non-extendible by Taq DNA polymerase enzyme, and is labeled with a reporter fluorescent dye and a quencher fluorescent dye. Any laser-induced emission from the reporter dye is quenched by the quenching dye when the two dyes are located close together as they are on the probe. During the amplification reaction, the Taq DNA polymerase enzyme cleaves the probe in a template-dependent manner. The resultant probe fragments disassociate in solution, and signal from the released reporter dye is free from the quenching effect of the second fluorophore. One molecule of reporter dye is liberated for each new molecule synthesized, and detection of the unquenched reporter dye provides the basis for quantitative interpretation of the data.

[0049] TaqMan® RT-PCR can be performed using commercially available equipment, such as, for example, ABI PRISM 7700™ Sequence Detection System™ (Perkin-Elmer-Applied Biosystems, Foster City, CA, USA), or Lightcycler (Roche Molecular Biochemicals, Mannheim, Germany). In a preferred embodiment, the 5' nuclease procedure is run on a real-time quantitative PCR device such as the ABI PRISM 7700™ Sequence Detection System™. The system consists of a thermocycler, laser, charge-coupled device (CCD), camera and computer. The system amplifies samples in a 96-well format on a thermocycler. During amplification, laser-induced fluorescent signal is collected in real-time

through fiber optics cables for all 96 wells, and detected at the CCD. The system includes software for running the instrument and for analyzing the data.

[0050] 5'-Nuclease assay data are initially expressed as Ct, or the threshold cycle. As discussed above, fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The point when the fluorescent signal is first recorded as statistically significant is the threshold cycle (C_t).

[0051] To minimize errors and the effect of sample-to-sample variation, RT-PCR is usually performed using an internal standard. The ideal internal standard is expressed at a constant level among different tissues, and is unaffected by the experimental treatment. RNAs most frequently used to normalize patterns of gene expression are mRNAs for the housekeeping genes glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and β -actin.

[0052] A more recent variation of the RT-PCR technique is the real time quantitative PCR, which measures PCR product accumulation through a dual-labeled fluorogenic probe (i.e., TaqMan® probe). Real time PCR is compatible both with quantitative competitive PCR, where internal competitor for each target sequence is used for normalization, and with quantitative comparative PCR using a normalization gene contained within the sample, or a housekeeping gene for RT-PCR. For further details see, e.g. Held *et al.*, *Genome Research* 6:986-994 (1996).

[0053] According to one aspect of the present invention, PCR primers and probes are designed based upon intron sequences present in the gene to be amplified. In this embodiment, the first step in the primer/probe design is the delineation of intron sequences within the genes. This can be done by publicly available software, such as the DNA BLAT software developed by Kent, W.J., *Genome Res.* 12(4):656-64 (2002), or by the BLAST software including its variations. Subsequent steps follow well established methods of PCR primer and probe design.

[0054] In order to avoid non-specific signals, it is important to mask repetitive sequences within the introns when designing the primers and probes. This can be easily accomplished by using the Repeat Masker program available on-line through the Baylor College of Medicine, which screens DNA sequences against a library of repetitive elements and returns a query sequence in which the repetitive elements are masked. The masked intron sequences can then be used to design primer and probe sequences using any commercially or

otherwise publicly available primer/probe design packages, such as Primer Express (Applied Biosystems); MGB assay-by-design (Applied Biosystems); Primer3 (Steve Rozen and Helen J. Skaletsky (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp 365-386)

[0055] The most important factors considered in PCR primer design include primer length, melting temperature (T_m), and G/C content, specificity, complementary primer sequences, and 3'-end sequence. In general, optimal PCR primers are generally 17-30 bases in length, and contain about 20-80%, such as, for example, about 50-60% G+C bases. T_m 's between 50 and 80 °C, e.g. about 50 to 70 °C are typically preferred.

[0056] For further guidelines for PCR primer and probe design see, e.g. Dieffenbach, C.W. *et al.*, "General Concepts for PCR Primer Design" in: *PCR Primer, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, 1995, pp. 133-155; Innis and Gelfand, "Optimization of PCRs" in: *PCR Protocols, A Guide to Methods and Applications*, CRC Press, London, 1994, pp. 5-11; and Plasterer, T.N. Primerselect: Primer and probe design. *Methods Mol. Biol.* 70:520-527 (1997), the entire disclosures of which are hereby expressly incorporated by reference.

3. Microarrays

[0057] Differential gene expression can also be identified, or confirmed using the microarray technique. Thus, the expression profile of breast cancer-associated genes can be measured in either fresh or paraffin-embedded tumor tissue, using microarray technology. In this method, polynucleotide sequences of interest (including cDNAs and oligonucleotides) are plated, or arrayed, on a microchip substrate. The arrayed sequences are then hybridized with specific DNA probes from cells or tissues of interest. Just as in the RT-PCR method, the source of mRNA typically is total RNA isolated from human tumors or tumor cell lines, and corresponding normal tissues or cell lines. Thus RNA can be isolated from a variety of primary tumors or tumor cell lines. If the source of mRNA is a primary tumor, mRNA can be extracted, for example, from frozen or archived paraffin-embedded and fixed (e.g. formalin-fixed) tissue samples, which are routinely prepared and preserved in everyday clinical practice.

[0058] In a specific embodiment of the microarray technique, PCR amplified inserts of cDNA clones are applied to a substrate in a dense array. Preferably at least 10,000 nucleotide sequences are applied to the substrate. The microarrayed genes, immobilized on the microchip at 10,000 elements each, are suitable for hybridization under stringent conditions. Fluorescently labeled cDNA probes may be generated through incorporation of fluorescent nucleotides by reverse transcription of RNA extracted from tissues of interest. Labeled cDNA probes applied to the chip hybridize with specificity to each spot of DNA on the array. After stringent washing to remove non-specifically bound probes, the chip is scanned by confocal laser microscopy or by another detection method, such as a CCD camera. Quantitation of hybridization of each arrayed element allows for assessment of corresponding mRNA abundance. With dual color fluorescence, separately labeled cDNA probes generated from two sources of RNA are hybridized pairwise to the array. The relative abundance of the transcripts from the two sources corresponding to each specified gene is thus determined simultaneously. The miniaturized scale of the hybridization affords a convenient and rapid evaluation of the expression pattern for large numbers of genes. Such methods have been shown to have the sensitivity required to detect rare transcripts, which are expressed at a few copies per cell, and to reproducibly detect at least approximately two-fold differences in the expression levels (Schena *et al.*, *Proc. Natl. Acad. Sci. USA* 93(2):106-149 (1996)). Microarray analysis can be performed by commercially available equipment, following manufacturer's protocols, such as by using the Affymetrix GenChip technology, or Incyte's microarray technology.

[0059] The development of microarray methods for large-scale analysis of gene expression makes it possible to search systematically for molecular markers of cancer classification and outcome prediction in a variety of tumor types.

4. Serial Analysis of Gene Expression (SAGE)

[0060] Serial analysis of gene expression (SAGE) is a method that allows the simultaneous and quantitative analysis of a large number of gene transcripts, without the need of providing an individual hybridization probe for each transcript. First, a short sequence tag (about 10-14 bp) is generated that contains sufficient information to uniquely identify a transcript, provided that the tag is obtained from a unique position within each transcript. Then, many transcripts are linked together to form long serial molecules, that can

be sequenced, revealing the identity of the multiple tags simultaneously. The expression pattern of any population of transcripts can be quantitatively evaluated by determining the abundance of individual tags, and identifying the gene corresponding to each tag. For more details see, e.g. Velculescu *et al.*, *Science* 270:484-487 (1995); and Velculescu *et al.*, *Cell* 88:243-51 (1997).

5. MassARRAY Technology

[0061] The MassARRAY (Sequenom, San Diego, California) technology is an automated, high-throughput method of gene expression analysis using mass spectrometry (MS) for detection. According to this method, following the isolation of RNA, reverse transcription and PCR amplification, the cDNAs are subjected to primer extension. The cDNA-derived primer extension products are purified, and dispensed on a chip array that is pre-loaded with the components needed for MALTI-TOF MS sample preparation. The various cDNAs present in the reaction are quantitated by analyzing the peak areas in the mass spectrum obtained.

6. Gene Expression Analysis by Massively Parallel Signature Sequencing (MPSS)

[0062] This method, described by Brenner *et al.*, *Nature Biotechnology* 18:630-634 (2000), is a sequencing approach that combines non-gel-based signature sequencing with *in vitro* cloning of millions of templates on separate 5 μm diameter microbeads. First, a microbead library of DNA templates is constructed by *in vitro* cloning. This is followed by the assembly of a planar array of the template-containing microbeads in a flow cell at a high density (typically greater than 3×10^6 microbeads/cm²). The free ends of the cloned templates on each microbead are analyzed simultaneously, using a fluorescence-based signature sequencing method that does not require DNA fragment separation. This method has been shown to simultaneously and accurately provide, in a single operation, hundreds of thousands of gene signature sequences from a yeast cDNA library.

7. Immunohistochemistry

[0063] Immunohistochemistry methods are also suitable for detecting the expression levels of the prognostic markers of the present invention. Thus, antibodies or antisera, preferably polyclonal antisera, and most preferably monoclonal antibodies specific for each marker are used to detect expression. The antibodies can be detected by direct labeling of the antibodies themselves, for example, with radioactive labels, fluorescent labels, hapten labels such as, biotin, or an enzyme such as horse radish peroxidase or alkaline phosphatase. Alternatively, unlabeled primary antibody is used in conjunction with a labeled secondary antibody, comprising antisera, polyclonal antisera or a monoclonal antibody specific for the primary antibody. Immunohistochemistry protocols and kits are well known in the art and are commercially available.

8. Proteomics

[0064] The term "proteome" is defined as the totality of the proteins present in a sample (e.g. tissue, organism, or cell culture) at a certain point of time. Proteomics includes, among other things, study of the global changes of protein expression in a sample (also referred to as "expression proteomics"). Proteomics typically includes the following steps: (1) separation of individual proteins in a sample by 2-D gel electrophoresis (2-D PAGE); (2) identification of the individual proteins recovered from the gel, e.g. by mass spectrometry or N-terminal sequencing, and (3) analysis of the data using bioinformatics. Proteomics methods are valuable supplements to other methods of gene expression profiling, and can be used, alone or in combination with other methods, to detect the products of the prognostic markers of the present invention.

9. Improved Method for Isolation of Nucleic Acid from Archived Tissue Specimens

[0065] In the first step of the method of the invention, total RNA is extracted from the source material of interest, including fixed, paraffin-embedded tissue specimens, and purified sufficiently to act as a substrate in an enzyme assay. While extraction of total RNA can be performed by any method known in the art, in a particular embodiment, the invention relies on an improved method for the isolation of nucleic acid from archived, e.g. fixed, paraffin-embedded tissue specimens (FPET).

[0066] Measured levels of mRNA species are useful for defining the physiological or pathological status of cells and tissues. RT-PCR (which is discussed above) is one of the most sensitive, reproducible and quantitative methods for this "gene expression profiling". Paraffin-embedded, formalin-fixed tissue is the most widely available material for such studies. Several laboratories have demonstrated that it is possible to successfully use fixed-paraffin-embedded tissue (FPET) as a source of RNA for RT-PCR (Stanta *et al.*, *Biotechniques* 11:304-308 (1991); Stanta *et al.*, *Methods Mol. Biol.* 86:23-26 (1998); Jackson *et al.*, *Lancet* 1:1391 (1989); Jackson *et al.*, *J. Clin. Pathol.* 43:499-504 (1999); Finke *et al.*, *Biotechniques* 14:448-453 (1993); Goldsworthy *et al.*, *Mol. Carcinog.* 25:86-91 (1999); Stanta and Bonin, *Biotechniques* 24:271-276 (1998); Godfrey *et al.*, *J. Mol. Diagnostics* 2:84 (2000); Specht *et al.*, *J. Mol. Med.* 78:B27 (2000); Specht *et al.*, *Am. J. Pathol.* 158:419-429 (2001)). This allows gene expression profiling to be carried out on the most commonly available source of human biopsy specimens, and therefore potentially to create new valuable diagnostic and therapeutic information.

[0067] The most widely used protocols utilize hazardous organic solvents, such as xylene, or octane (Finke *et al.*, *supra*) to dewax the tissue in the paraffin blocks before nucleic acid (RNA and/or DNA) extraction. Obligatory organic solvent removal (e.g. with ethanol) and rehydration steps follow, which necessitate multiple manipulations, and addition of substantial total time to the protocol, which can take up to several days. Commercial kits and protocols for RNA extraction from FPET [MasterPure™ Complete DNA and RNA Purification Kit (EPICENTRE®, Madison, WI); Paraffin Block RNA Isolation Kit (Ambion, Inc.) and RNeasy™ Mini kit (Qiagen, Chatsworth, CA)] use xylene for deparaffinization, in procedures which typically require multiple centrifugations and ethanol buffer changes, and incubations following incubation with xylene.

[0068] The method that can be used in the present invention provides an improved nucleic acid extraction protocol that produces nucleic acid, in particular RNA, sufficiently intact for gene expression measurements. The key step in this improved nucleic acid extraction protocol is the performance of dewaxing without the use of any organic solvent, thereby eliminating the need for multiple manipulations associated with the removal of the organic solvent, and substantially reducing the total time to the protocol. According to the improved method, wax, e.g. paraffin is removed from wax-embedded tissue samples by

incubation at 65-75 °C in a lysis buffer that solubilizes the tissue and hydrolyzes the protein, following by cooling to solidify the wax.

[0069] Figure 2 shows a flow chart of the improved RNA extraction protocol used herein in comparison with a representative commercial method, using xylene to remove wax. The times required for individual steps in the processes and for the overall processes are shown in the chart. As shown, the commercial process requires approximately 50% more time than the improved process used in performing the methods of the invention.

[0070] The lysis buffer can be any buffer known for cell lysis. It is, however, preferred that oligo-dT-based methods of selectively purifying polyadenylated mRNA not be used to isolate RNA for the present invention, since the bulk of the mRNA molecules are expected to be fragmented and therefore will not have an intact polyadenylated tail, and will not be recovered or available for subsequent analytical assays. Otherwise, any number of standard nucleic acid purification schemes can be used. These include chaotrope and organic solvent extractions, extraction using glass beads or filters, salting out and precipitation based methods, or any of the purification methods known in the art to recover total RNA or total nucleic acids from a biological source.

[0071] Lysis buffers are commercially available, such as, for example, from Qiagen, Epicentre, or Ambion. A preferred group of lysis buffers typically contains urea, and Proteinase K or other protease. Proteinase K is very useful in the isolation of high quality, undamaged DNA or RNA, since most mammalian DNases and RNases are rapidly inactivated by this enzyme, especially in the presence of 0.5 - 1% sodium dodecyl sulfate (SDS). This is particularly important in the case of RNA, which is more susceptible to degradation than DNA. While DNases require metal ions for activity, and can therefore be easily inactivated by chelating agents, such as EDTA, there is no similar co-factor requirement for RNases.

[0072] Cooling and resultant solidification of the wax permits easy separation of the wax from the total nucleic acid, which can be conveniently precipitated, e.g. by isopropanol. Further processing depends on the intended purpose. If the proposed method of RNA analysis is subject to bias by contaminating DNA in an extract, the RNA extract can be further treated, e.g. by DNase, post purification to specifically remove DNA while preserving RNA. For example, if the goal is to isolate high quality RNA for subsequent RT-PCR

amplification, nucleic acid precipitation is followed by the removal of DNA, usually by DNase treatment. However, DNA can be removed at various stages of nucleic acid isolation, by DNase or other techniques well known in the art.

[0073] While the advantages of the improved nucleic acid extraction discussed above are most apparent for the isolation of RNA from archived, paraffin embedded tissue samples, the wax removal step of the present invention, which does not involve the use of an organic solvent, can also be included in any conventional protocol for the extraction of total nucleic acid (RNA and DNA) or DNA only.

[0074] By using heat followed by cooling to remove paraffin, the improved process saves valuable processing time, and eliminates a series of manipulations, thereby potentially increasing the yield of nucleic acid.

10. 5'-multiplexed Gene Specific Priming of Reverse Transcription

[0075] RT-PCR requires reverse transcription of the test RNA population as a first step. The most commonly used primer for reverse transcription is oligo-dT, which works well when RNA is intact. However, this primer will not be effective when RNA is highly fragmented as is the case in FPE tissues.

[0076] The present invention includes the use of gene specific primers, which are roughly 20 bases in length with a T_m optimum between about 58 °C and 60 °C. These primers will also serve as the reverse primers that drive PCR DNA amplification.

[0077] An alternative approach is based on the use of random hexamers as primers for cDNA synthesis. However, we have experimentally demonstrated that the method of using a multiplicity of gene-specific primers is superior over the known approach using random hexamers.

11. Normalization Strategy

[0078] An important aspect of the present invention is to use the measured expression of certain genes by EGFR-expressing cancer tissue to provide information about the patient's likely response to treatment with an EGFR-inhibitor. For this purpose it is necessary to correct for (normalize away) both differences in the amount of RNA assayed and variability in the quality of the RNA used. Therefore, the assay typically measures and incorporates the expression of certain normalizing genes, including well known housekeeping genes, such as GAPDH and Cyp1. Alternatively or in addition, normalization

can be based on the mean or median signal (Ct in the case of RT-PCR) of all of the assayed genes or a large subset thereof (global normalization approach). On a gene-by-gene basis, measured normalized amount of a patient tumor mRNA is compared to the amount found in a reference set of cancer tissue of the same type (e.g. head and neck cancer, colon cancer, etc.). The number (N) of cancer tissues in this reference set should be sufficiently high to ensure that different reference sets (as a whole) behave essentially the same way. If this condition is met, the identity of the individual cancer tissues present in a particular set will have no significant impact on the relative amounts of the genes assayed. Usually, the cancer tissue reference set consists of at least about 30, preferably at least about 40 different FPE cancer tissue specimens. Unless noted otherwise, normalized expression levels for each mRNA/tested tumor/patient will be expressed as a percentage of the expression level measured in the reference set. More specifically, the reference set of a sufficiently high number (e.g. 40) of tumors yields a distribution of normalized levels of each mRNA species. The level measured in a particular tumor sample to be analyzed falls at some percentile within this range, which can be determined by methods well known in the art. Below, unless noted otherwise, reference to expression levels of a gene assume normalized expression relative to the reference set although this is not always explicitly stated.

12. EGFR Inhibitors

[0079] The epidermal growth factor receptor (EGFR) family (which includes EGFR, erb-B2, erb-B3, and erb-B4) is a family of growth factor receptors that are frequently activated in epithelial malignancies. Thus, the epidermal growth factor receptor (EGFR) is known to be active in several tumor types, including, for example, ovarian cancer, pancreatic cancer, non-small cell lung cancer, breast cancer, colon cancer and head and neck cancer. Several EGFR inhibitors, such as ZD1839 (also known as gefitinib or Iressa); and OSI774 (Erlotinib, Tarceva™), are promising drug candidates for the treatment of EGFR-expressing cancer.

[0080] Iressa, a small synthetic quinazoline, competitively inhibits the ATP binding site of EGFR, a growth-promoting receptor tyrosine kinase, and has been in Phase III clinical trials for the treatment of non-small-cell lung carcinoma. Another EGFR inhibitor, [agr]cyano-[bgr]methyl-N-[(trifluoromethoxy)phenyl]-propenamide (LFM-A12), has been

shown to inhibit the proliferation and invasiveness of EGFR positive human breast cancer cells.

[0081] Cetuximab is a monoclonal antibody that blocks the EGFR and EGFR-dependent cell growth. It is currently being tested in phase III clinical trials.

[0082] Tarceva™ has shown promising indications of anti-cancer activity in patients with advanced ovarian cancer, and non-small cell lung and head and neck carcinomas.

[0083] The present invention provides valuable tools to predict whether an EGFR-positive tumor is likely to respond to treatment with an EGFR-inhibitor.

[0084] Recent publications further confirm the involvement of EGFR in gastrointestinal (e.g. colon) cancer, and associate its expression with poor survival. See, e.g. Khorana *et al.*, *Proc. Am. Soc. Clin. Oncol* 22:317 (2003).

[0085] While the listed examples of EGFR inhibitors are small organic molecules, the findings of the present invention are equally applicable to other EGFR inhibitors, including, without limitation, anti-EGFR antibodies, antisense molecules, small peptides, etc.

[0086] Further details of the invention will be apparent from the following non-limiting Examples.

Example 1

A Phase II Study of Gene Expression in Head and Neck Tumors

[0087] A gene expression study was designed and conducted with the primary goal to molecularly characterize gene expression in paraffin-embedded, fixed tissue samples of head and neck cancer patients who responded or did not respond to treatment with an EGFR inhibitor. The results are based on the use of five different EGFR inhibitor drugs.

Study design

[0088] Molecular assays were performed on paraffin-embedded, formalin-fixed head and neck tumor tissues obtained from 14 individual patients diagnosed with head and neck cancer. Patients were included in the study only if histopathologic assessment, performed as described in the Materials and Methods section, indicated adequate amounts of tumor tissue.

Materials and Methods

[0089] Each representative tumor block was characterized by standard histopathology for diagnosis, semi-quantitative assessment of amount of tumor, and tumor grade. A total of 6 sections (10 microns in thickness each) were prepared and placed in two Costar Brand Microcentrifuge Tubes (Polypropylene, 1.7 mL tubes, clear; 3 sections in each tube). If the tumor constituted less than 30% of the total specimen area, the sample may have been crudely dissected by the pathologist, using gross microdissection, putting the tumor tissue directly into the Costar tube.

[0090] If more than one tumor block was obtained as part of the surgical procedure, all tumor blocks were subjected to the same characterization, as described above, and the block most representative of the pathology was used for analysis.

Gene Expression Analysis

[0091] mRNA was extracted and purified from fixed, paraffin-embedded tissue samples, and prepared for gene expression analysis as described above.

[0092] Molecular assays of quantitative gene expression were performed by RT-PCR, using the ABI PRISM 7900™ Sequence Detection System™ (Perkin-Elmer-Applied Biosystems, Foster City, CA, USA). ABI PRISM 7900™ consists of a thermocycler, laser, charge-coupled device (CCD), camera and computer. The system amplifies samples in a 384-well format on a thermocycler. During amplification, laser-induced fluorescent signal is collected in real-time through fiber optics cables for all 384 wells, and detected at the CCD. The system includes software for running the instrument and for analyzing the data.

Analysis and Results

[0093] Tumor tissue was analyzed for 185 cancer-related genes and 7 reference genes. The threshold cycle (CT) values for each patient were normalized based on the mean of all genes for that particular patient. Clinical outcome data were available for all patients.

[0094] Outcomes were classified as either response or no response. The results were analyzed in two different ways using two different criteria for response: partial response, or clinical benefit. The latter criterion combines partial or complete response with stable disease (minimum 3 months). In this study, there were no complete responses, four cases of partial response and two cases of disease stabilization.

[0095] We evaluated the relationship between gene expression and partial response by logistic regression and have identified the following genes as significant ($p < 0.15$), as indicated in the attached Table 1. The logistic model provides a means of predicting the probability (Pr) of a subject as being either a partial responder or not. The following equation defined the expression threshold for response.

$$\text{Pr}(\text{Response}) = \frac{1}{1 + e^{\text{Intercept} + \text{Slope} \times \text{Reference Normalized CT}}} \quad \text{and} \quad \text{Pr}(\text{No Response}) = 1 - \text{Pr}(\text{Response})$$

[0096] In Table 1, the term "negative" indicates that greater expression of the gene decreased likelihood of response to treatment with EGFR inhibitor, and "positive" indicates that increased expression of the gene increased likelihood of response to EGFR inhibitor. Results from analysis of head and neck cancer patient data using clinical benefit criteria are shown in Table 2.

[0097] Overall increased expression of the following genes correlated with resistance of head and neck cancer to EGFR inhibitor treatment: A-Catenin; AKT1; AKT2; APC; Bax; B-Catenin; BTC; CCNA2; CCNE1; CCNE2; CD105; CD44v3; CD44v6; CD68; CEACAM6; Chk2; cMet; COX2; cripto; DCR3; DIABLO; DPYD; DR5; EDN1 endothelin; EGFR; EIF4E; ERBB4; ERK1; fas; FRP1; GRO1; HB-EGF; HER2; IGF1R; IRS1; ITGA3; KRT17; LAMC2; MTA1; NMYC; PAI1; PDGFA; PGK1; PTPD1; RANBP2; SPRY2; TP53BP1; and VEGFC; and increased expression of the following genes correlated with response of head and neck cancer to EGFR inhibitor treatment: CD44s; CD82; CGA; CTSL; EGFRd27; IGFBP3; p27; P53; RB1; TIMP2; and YB-1.

Example 2

A Phase II Study of Gene Expression in Colon Cancer

[0098] In a study analogous to the study of head and neck cancer patients described in Example 1, gene expression markers were sought that correlate with increased or decreased likelihood of colon cancer response to EGFR inhibitors. Sample preparation and handling and gene expression and data analysis were performed as in Example 1.

[0099] Twenty-three colon adenocarcinoma patients in all were studied, using a 192 gene assay. 188 of the 192 genes were expressed above the limit of detection. Both pathological and clinical responses were evaluated. Following treatment with EGFR

inhibitor, three patients were determined to have had a partial response, five to have stable disease and fifteen to have progressive disease.

[0100] Table 3 shows the results obtained using the partial response criterion.

[0101] Results from analysis of colon cancer patient data using clinical benefit criteria are shown in Table 4.

[0102] Overall, increased expression of the following genes correlated with resistance of colon cancer to EGFR inhibitor treatment: CA9; CD134; CD44E; CD44v3; CD44v6; CDC25B; CGA; DR5; GRO1; KRT17; LAMC2; P14ARF; PDGFB; PLAUR; PPARG; RASSF1; RIZ1; Src; TFRC; and UPA, and increased expression of the following genes correlated with sensitivity of colon cancer to EGFR inhibitor treatment: CD44s; CD82; CGA; CTSL; EGFRd27; IGFBP3; p27; P53; RB1; TIMP2; and YB-1.

[0103] Finally, it is noteworthy that increased expression of the following genes correlated with resistance to EGFR inhibitor treatment in both head and neck and colon cancer: CD44v3; CD44v6; DR5; GRO1; KRT17; LAMC2.

[0104] In similar experiments, the elevated expression of LAMC2, B-Catenin, Bax, GRO1, Fas, or ITGA3 in EGFR-positive head and neck cancer was determined to be an indication that the patient is not likely to respond well to treatment with an EGFR inhibitor. On the other hand, elevated expression of YB-1, PTEN, CTSL, P53, STAT3, ITGB3, IGFBP3, RPLPO or p27 in EGFR-positive head and neck cancer was found to be an indication that the patient is likely to respond to EGFR inhibitor treatment.

[0105] In another set of similar experiments, elevated expression of the following genes in EGFR-expressing colon cancer correlated with positive response to treatment: BAK; BCL2; BRAF; BRK; CCND3; CD9; ER2; ERBB4; EREG; ERK1; FRP1. Elevated expression of the following genes in EGFR-expressing colon cancer correlated with resistance to treatment: APN; CA9; CCND1; CDC25B; CD134; LAMC2; PDGFB; CD44v6; CYP1; DR5; GAPDH; IGFBP2; PLAUR; RASSF1; UPA.

[0106] All references cited throughout the specification are hereby expressly incorporated by reference.

[0107] Although the present invention is illustrated with reference to certain embodiments, it is not so limited. Modifications and variations are possible without diverting from the spirit of the invention. All such modifications and variations, which will

be apparent to those skilled in the art, are specifically within the scope of the present invention. While the specific examples disclosed herein concern head and neck cancer and colon cancer, the methods of the present invention are generally applicable and can be extended to all EGFR-expressing cancers, and such general methods are specifically intended to be within the scope herein.

Table 1: Partial Response Genes for Head and Neck Study

Gene Name	Response	Logistic Discriminat Function		R2	Likelihood Ratio Test
		Intercept	Slope		P Value
cMet	Negative	26.5168713	4.57143179	0.6662	0.0011
LAMC2	Negative	5.29706425	1.28137295	0.6155	0.0017
ITGA3	Negative	22.6008544	3.17707499	0.5063	0.0044
CD44v6	Negative	6.92255059	4.3069909	0.492	0.005
B-Catenin	Negative	7.85913706	2.52965454	0.4805	0.0055
PDGFA	Negative	6.0016358	1.10386463	0.4318	0.0085
GRO1	Negative	8.37646635	1.74815793	0.4146	0.0099
ERK1	Negative	6.14712633	1.64819007	0.4024	0.0111
CD44v3	Negative	5.95094528	3.36594473	0.3451	0.0186
Bax	Negative	5.34006632	1.19383253	0.3361	0.0202
CGA	Positive	-78.121148	-10.503757	0.3266	0.0221
fas	Negative	7.27491015	1.38464586	0.3251	0.0224
IGFBP3	Positive	-2.1529531	-2.7937517	0.3097	0.0258
MTA1	Negative	6.07167277	1.23786874	0.3072	0.0264
YB-1	Positive	1.73598983	-4.0859174	0.2814	0.0336
DR5	Negative	9.0550349	1.46349944	0.2703	0.0373
APC	Negative	5.775003	1.88324269	0.2512	0.0447
ERBB4	Negative	11.9466285	1.58606697	0.2357	0.0518
CD68	Negative	3.60605487	1.0645631	0.2319	0.0537
cripto	Negative	19.5004373	2.64909385	0.2251	0.0574
P53	Positive	-4.1976158	-1.5541169	0.2208	0.0598
VEGFC	Negative	6.33634489	0.90613473	0.2208	0.0598
A-Catenin	Negative	4.41215235	1.7591194	0.2199	0.0603
COX2	Negative	8.00968996	1.27597736	0.202	0.0718
CD82	Positive	-1.8999985	-1.171157	0.1946	0.0772
PAI1	Negative	2.94777884	0.97480364	0.1944	0.0774
AKT2	Negative	2.45598587	1.64608189	0.1889	0.0817
HER2	Negative	4.25059223	0.97748483	0.1845	0.0853
DIABLO	Negative	17.035069	2.93939741	0.1809	0.0884
p27	Positive	-1.9798519	-1.9041142	0.1792	0.09
RANBP2	Negative	2.85994976	0.41878666	0.1757	0.0931
EIF4E	Negative	2.91202768	0.56099402	0.1722	0.0965
EDN1 endothelin	Negative	6.06858911	0.87185553	0.1688	0.0998
IGF1R	Negative	6.14387144	1.68865744	0.1674	0.1012
AKT1	Negative	5.02676228	1.50585593	0.1659	0.1028
CCNA2	Negative	3.95684559	0.63089954	0.184	0.1033
HB-EGF	Negative	5.1019713	0.70368632	0.1627	0.1061
TIMP2	Positive	2.58975885	-1.0832648	0.1625	0.1064

Gene Nam	Response	Logistic Discriminat Function		R2	Likelihood Ratio Test
		Intercept	Slope		P Valu
EGFRd27	Positive	-38.789016	-5.2513587	0.1607	0.1083
Chk2	Negative	6.8797175	1.21671205	0.1581	0.1112
IRS1	Negative	12.0545078	1.59632708	0.1578	0.1115
FRP1	Negative	3.38233862	0.49053452	0.1569	0.1126
CCNE2	Negative	5.78828731	1.11609099	0.1566	0.1129
SPRY2	Negative	4.68447069	0.86747803	0.1552	0.1145
KRT17	Negative	0.34280253	0.412313	0.151	0.1195
DPYD	Negative	2.78071456	0.78918833	0.1504	0.1202
CD105	Negative	3.13613733	0.51406689	0.1391	0.1351
TP53BP1	Negative	3.18676588	0.58622276	0.1361	0.1395
PTPD1	Negative	5.85217342	1.08545385	0.1357	0.1401
CTSL	Positive	-2.2283797	-1.4833372	0.1354	0.1405

Table 2: Clinical Benefit Genes for Head and Neck Study

Gene Name	Response	Logistic Discriminat Function		R ²	Likelihood Ratio T _{st}
		Intercept	Slope		P Value
cMet.2	Negative	23.583252	4.4082875	0.6444	0.0007
GRO1.2	Negative	10.10717	2.46904056	0.5388	0.0019
A-Catenin.2	Negative	5.13298651	2.60834812	0.3628	0.0107
AKT1.3	Negative	7.7652606	2.83068092	0.3044	0.0194
DCR3.3	Negative	10.2957141	1.85012996	0.293	0.0219
B-Catenin.3	Negative	4.21267279	1.5417788	0.2791	0.0252
EDN1 endothelin.1	Negative	6.83022814	1.14550062	0.2758	0.0261
CCNE1.1	Negative	7.43731399	1.21270723	0.2661	0.0289
LAMC2.2	Negative	1.79659862	0.56623898	0.2498	0.0342
CD44v6.1	Negative	2.55050577	1.87838162	0.2071	0.0539
DIABLO.1	Negative	16.5051841	2.99910512	0.2066	0.0542
CD44v3.2	Negative	3.02492619	2.05469571	0.2002	0.058
NMYC.2	Negative	23.2010327	3.20767305	0.1955	0.061
CD82.3	Positive	-2.7521937	-1.1692268	0.188	0.0662
RANBP2.3	Negative	2.02076788	0.42173233	0.1807	0.0718
RB1.1	Positive	-5.7352964	-1.7540651	0.1761	0.0754
HER2.3	Negative	3.87564158	1.11486016	0.1732	0.0779
MTA1.1	Negative	3.9020256	0.92255645	0.1628	0.0874
CGA.3	Positive	-41.909839	-5.5686182	0.1619	0.0883
CEACAM6.1	Negative	1.66596967	0.59307792	0.1602	0.0899
PTPD1.2	Negative	5.51242763	1.18616068	0.1601	0.0901
ERK1.3	Negative	2.4144706	0.72072834	0.154	0.0964
Bax.1	Negative	2.91338256	0.76334619	0.152	0.0987
STMY3.3	Positive	-0.9946728	-0.6053981	0.1483	0.1028
COX2.1	Negative	5.79279616	1.0312018	0.1478	0.1034
EIF4E.1	Negative	2.08005397	0.55985052	0.1468	0.1045
YB-1.2	Positive	0.45158771	-2.2935538	0.1426	0.1096
fas.1	Negative	4.05538424	0.8686042	0.1397	0.1134
PDGFA.3	Negative	2.43388275	0.53168307	0.1371	0.1168
FRP1.3	Negative	2.17320245	0.41529609	0.137	0.1169
PGK1.1	Negative	1.86416703	1.92395917	0.1338	0.1212
AKT2.3	Negative	1.45131206	1.43341036	0.1281	0.1294
BTC.3	Negative	12.1153734	1.67411928	0.1281	0.1294
APC.4	Negative	2.50791938	0.92506412	0.128	0.1296
CCNE2.2	Negative	3.98727145	0.89372321	0.1267	0.1315
OPN, osteopontin.3	Positive	-0.522697	-0.5069258	0.1225	0.1382
ITGA3.2	Negative	2.23381763	0.3800099	0.1203	0.1417
KRT17.2	Negative	-0.4861169	0.43917211	0.1184	0.1449

Gene Name	Response	Logistic Discriminat Function		R ²	Likelihood Ratio Test
		Interc pt	Slope		P Value
CD44s.1	Positive	-0.9768133	-0.8896223	0.118	0.1456
EGFR.2	Negative	0.43258354	0.46719029	0.1162	0.1487

Table 3: Partial Response Genes for Colon Study

Gene Name	Response	Logistic Discriminat Function		R ²	Likelihood Ratio Test
		Intercept	Slope		P Value
Bclx_2	Positive	2.04896151	-2.1025144	0.172	0.0801
BRAF_2	Positive	-2.5305788	-3.0987684	0.2532	0.0337
BRK_2	Positive	-2.6096501	-1.577388	0.2998	0.0209
CA9_3	Negative	2.65287578	0.83720397	0.2758	0.0267
Cad17_1	Positive	-0.0419396	-1.8773242	0.2096	0.0533
CCND3_1	Positive	-1.014844	-5.1111617	0.348	0.0128
CCNE1_1	Positive	-6.5821701	-0.8939912	0.1914	0.0648
CCNE2_2	Positive	26.1675642	-1.0709109	0.1707	0.0812
CD105_1	Positive	5.85359096	-1.2349006	0.1302	0.1278
CD134_2	Negative	-5.9286576	1.51119518	0.1212	0.1418
CD44v3_2	Negative	-1.8184898	1.12771829	0.2064	0.0552
CDC25B_1	Negative	10.4351019	1.59196005	0.2455	0.0365
DR5_2	Negative	-1.7399226	1.60177588	0.1759	0.0767
ErbB3_1	Positive	3.65681435	-0.760436	0.1222	0.1401
EREG_1	Positive	-2.3409861	-1.1217612	0.2542	0.0333
GPC3_1	Positive	4.03889935	-1.9097648	0.3752	0.0097
GRO1.2	Negative	2.77545378	0.74734483	0.124	0.1359
GUS_1	Positive	8.29578416	-1.9015759	0.2105	0.0529
HGF_4	Positive	5.10609383	-1.1947949	0.2361	0.0403
ID1_1	Positive	10.6703203	-1.654146	0.216	0.0498
ITGB3_1	Positive	0.79232612	-0.827508	0.3321	0.015
KRT17_2	Negative	5.93738146	0.93514633	0.2133	0.0513
LAMC2_2	Negative	-0.3325052	1.41542034	0.2475	0.0357
P14ARF_1	Negative	4.36456658	4.10859002	0.2946	0.022
PDGFB_3	Negative	-4.7055966	1.96517114	0.3299	0.0154
PLAUR_3	Negative	7.51817646	0.6862142	0.1534	0.0983
PTPD1_2	Positive	-11.659761	-1.2559081	0.1247	0.1362
RASSF1_3	Negative	6.60631474	0.9862129	0.1708	0.0811
RIZ1_2	Negative	2.83817546	0.86281199	0.1255	0.1349
Src_2	Negative	4.91364145	1.96089745	0.1324	0.1247
TFRC_3	Negative	-4.0754666	3.03617052	0.19	0.0658
TITF1_1	Positive	-1.8849815	-2.1890987	0.1349	0.1211
upa_3	Negative	4.1059421	1.14053848	0.1491	0.1032
XIAP_1	Positive	-16.296951	-2.9502191	0.2661	0.0295

Table 4: Clinical Benefit Genes for Colon Study

Gene Name	Response	Logistic Discriminat Function		R ²	Likelihood Ratio Test
		Intercept	Slope		P Value
Bak	Positive	-1.347937	-0.993212	0.1189	0.0602
BRK	Positive	-3.237705	-1.1479379	0.2567	0.0057
CD134	Negative	9.9358537	1.68440149	0.1927	0.0167
CD44E	Negative	3.188991	0.59091622	0.0958	0.0916
CD44v6	Negative	5.7352464	1.77571293	0.2685	0.0047
CDC25B	Negative	2.0664209	0.67140598	0.0783	0.1272
CGA	Negative	2.7903424	0.43834476	0.1035	0.0794
COX2	Positive	-1.262804	-0.4741852	0.0733	0.1398
DIABLO	Positive	-2.514199	-1.0753148	0.1028	0.0805
FRP1	Positive	-0.401936	-0.3555899	0.0937	0.0952
GPC3	Positive	-7.875276	-1.7437079	0.3085	0.0025
HER2	Positive	0.1228609	-0.5549133	0.073	0.1408
ITGB3	Positive	-1.593092	-0.5249778	0.1352	0.045
PPARG	Negative	8.6479233	1.36115361	0.1049	0.0774
PTPD1	Positive	-3.203607	-1.2049773	0.1356	0.0447
RPLPO	Positive	3.5110353	-1.030518	0.0752	0.135
STK15	Positive	-0.664989	-0.5936475	0.0873	0.1072
SURV	Positive	-1.409619	-0.6214924	0.074	0.1381
TERC	Positive	1.7755749	-0.5180083	0.1073	0.0742
TGFBR2	Positive	1.5172396	-0.9288498	0.0934	0.0957

Tables 5A - 5B

Gene	Accessin	Sequence	Seq. ID
A-Catenin	NM_00190	CGTTCCTCTATCTGCTATCCAGGCATGCTACAGCACCCCTGATGTGCGAGCCCTATAAGGCCAACAGGGACCT	1
AKT1	NM_00516	CGCTCTATGCGCTGAGATTGTGTCAGCCCTGGACTACCTGCACCTCGGAGAAAGAACGCTGGTGTACCGGGA	2
AKT2	NM_00162	TCCGCCACCTTCAACCTCAGGTACGTCGAGGTGCGACAAAGGTACTTCGATGATGAATTTACCGCC	3
APC	NM_00003	GGACAGCAGGAATGTCTTCTCCATACAGGTACGCGGAGCCAAATGGTTCAGAAACAAATCGAGTGGT	4
B-Catenin	NM_00190	GGCTCTGTGCGTACTGCTTCTCGGCTGGTGACAGGGAAGACATCATGAGCTGCCCTCTGCTCTTCTGTCATCTGA	5
Bak	NM_00118	ATCTCCACATCTACCTGAGGCGCAGGACGCTCTGGGTGGGGATGGTGGGTCTATGTTCC	6
Bax	NM_00432	CCGCGTGGACACAGACTCCCCCGAGAGGCTTTTCCGAGTGGCAGCTGACATGTTTCTGACGGCAA	7
Bclx	NM_00119	CTTTTGTGAACCTATGGAACAAATGCAGACGCGAGAGCCGAAAGGCCAGGAACGCTTCAACCGCTG	8
BRAF	NM_00433	CTTCCGACAGCAGATGAAGATATCGAAATCAATTTGGCAACGAGACCGATCTCATCAGCTCCCAATGTGCATATAA	9
BRK	NM_00597	GTGAGGAAGGTTTCAAAATGTGGAGTCTGCGTCCATACACGCGTGTCTCTCTTACTCCATCTGTTGTGTC	10
BTC	NM_00172	ATGGAGATGCCGCTTCGTGGTGGCGAGCAGCCCTCTGTGTGATGAAGGCTACATTTGAGCAAGGTGTGAGAG	11
CA9	NM_00121	ATCCTAGCCCTGTTTGGCTCTTTTGTGCTGTCACACGCTGCGTCTCTGTGCAGATGAGAAGGCAG	12
Cad17	NM_00406	GAAAGCCAAAGAACCGATCAATATATCCAGTTTAAGGCCAATCCTCTGCTGTGACTTTTGAACCTAACTGGGGA	13
CCNA2	NM_00123	CCATACCTCAAGTATTTGCCATCAGTATTGCTGGAGCTGCTTTTCAATTAGCACTCTACAGCTACGGGACAAAGCT	14
CCND3	NM_00176	CCTCTGTCTACAGATTATACCTTTGCCATGTACCCGCCATCCATGATCGCCACGGGCAGCATTTGGGGCTGCAGTG	15
CCNE1	NM_00123	AAAGAGATGATGACCGGTTTACCAAACTCAACGTCGAAGCCTCGGATTATTGCACCATCCAGAGGCTC	16
CCNE2	NM_05774	ATGCTGTGGCTCTTCTAAGTGGGCTTTCTTGACATGAGTGGTCAATATCTCTGCGACTATCCACACAGCGGAAAAA	17
CD105	NM_00111	GCAGGTGTCAGCAAGTATGATCAGCAATGAGCGGTTGTAATCTCTGCGAGCTCATCCACACAGCGGAAAAA	18
CD134	NM_00332	GCCAGTGCAGGAACAGGTCCAGCTTGATTCTGCTCTGCACTTAAGCTTTTCTCCAGGTGCGTGTGATT	19
CD44E	X55150	ATCACCGACAGCAGACAGAAATCCCTGCTACCAATATGGACTCCAGTACATAGTACAAAGCTTCAGCCTACTGCAAAATCCAAACACAGGT	20
CD44s	M59040	GACAAAGACAGTCCCTGGATCACCCAGCAGCAGACAGACAGAAATCCCTGCTACCAAGACCAAGACACATTCACCCCGAGT	21
CD44v3	AJ251595	CACATAAACAGAACAGGACTGGACCCAGTGAACCCCAAGCCATTCAATCCGGAAGTGTACTTCAG	22
CD44v6	AJ251595	CTCATACAGCCATCCAAATGAAAGAGGACACCAAGCCACAGGACAGTCTCTGGAGTGAATTTCTTCAACCCAA	23
CD68	NM_00125	GTGTTCCCGCTGTGTCACCTCCAAAGCCAGATTCAGATTCGAGTTCATGTACACACCCAGGGTGGAGGAG	24
CD82	NM_00223	GTGAGGCTCAGGTGAAGTGTGCGGCTGGTGTGCTTCTACAACCTGGACAGACAAAGCTGAGCTCATGAATCGCCCTGAGGTC	25
CD9	NM_00176	GGCGGTGAACAGTTTATCTAGACATCTGCCCCAAGAGGACGTACTCGAAACCTTCCACCGTG	26
CDC25B	NM_02187	AAACGAGCAGTTTGCCATCAGACGCTTCAGTCTATGCGGTGAGGCTGCTGGGCCACAGCCCGCTGCTCGGAACATCACCAAC	27
CEACAM6	NM_00248	CACAGCTCTACCTTCTAACCCTTCTGGAACCCACCCACCTCCAAAGCTCACTATTGAATCCAGGCCATTCAA	28
CGA	NM_00127	CTGAAGGAGCTCCAAAGACCTCGCTCCCAAGGCGCCAAAGGACATCCAGCTCACTATGATCCAGGCCATTCG	29
Chk2	NM_00719	ATGTGGAACCCACCTACTTGGCGCTGAAGTCTTGTTCTGTTGGAGTGTCTGGGTATACCGTGTGGACTG	30
dMet	NM_00024	GACATTTCCAGTCTGCAGTCAATGCTCTCTGCCCCACCTTTGTTCAAGTGTGGTGGTGGTGGTGGTGGTGGGAG	31
COX2	NM_00096	TCGACAGTTGGAAGCAGCTATGTTGACATCGATGCTGGAGCTGTATCTGCTGCTTCTGGTAGAAAGCCCTCGGC	32
cripto	NM_00321	GGTCTGCCCCATGACCTTGCTGCCCAAGAGTGTCCCTGTGTAAATGCTGGCACGGTCA	33
CTSL	NM_00191	GGGAGGCTTATCTCACTGAGTGAGCAGAAATCTGGTAGACTGCTTGGGCTCAAGGCAATGAAGGCTGCAATGG	34
DCR3	NM_01643	GACCAAGTCTTGAATGCTGCAGCAGAAAGTGAATGGCATCTCTGGAGAGCCCTACGGGTACAGGGAAGAC	35
DIABLO	NM_01988	CACAAATGGCGCTCTGAAGATGGCTGTGCGGACGCTAACTTCTTCAAGTACAGACAGTGTGTGT	36
DYPD	NM_00011	AGGACGCAAGGAGGTTTGTACCTGGCAGACTCGAGCTGAGGACCTGCCATGGCCCTGTGCTCAGTAAGGACTCGGCGGACATC	37
DR5	NM_00384	CTCTGACACAGTCTTCGATGACTTTCAGACTTGGGCTTGGCTTGAAGTGTGCTGCTGAGGAGTGGCCCTCATGG	38
EDN1_end	NM_00195	TGCGACCTGGACATCAITTTGGTCAACACTCCCGACAGCTGTTCGATGGACTTGAAGCCCTAGGTCCA	39
EGFR	NM_00522	TGTCGAGGACTTCCAGAACCACTTGGCAGCTGCCAAAGTGTGATGCTCAAGCTGTCCCAAT	40
EGFRd27	EGFRd27	GAGTGGGCTCTGGAGGAAAGAGTAAATATGTGGTACAGATACAGGCTCGTGGTCCGAGCCCTGTGG	41
EIF4E	NM_00196	GATCTAAGATGGCAGCTGCGAACCGGAAACCAACCCCTACTCTTAATCCCCGACTACAGAGAGAGGAAACGGAATCTAA	42
ErbB3	NM_00198	CGGTTATGTCATGCCAGATACACACTCAAGGCTCAAGGCTTCCCTCCCGGAAGGACCCCTTCTTTCAGTGGGTCTCAGTTTC	43
ERBB4	NM_00523	TGGCTCTTAATCAGTTTCTTACCTGCTTCCCTGCGGAAATTAACGATATTCGCGGACAAACCTTATGAGGATCGATATGCCCTTG	44
EREG	NM_00143	ATAACAAAGTGTAGCTCTGACATGAATGGCTATTTGTCATGGACAGTGCATCTATCTGGTGGACATGAGTCAAACTACTGCAGGTGTG	45
ERK1	Z11696	ACGGATCAGATGGAGGAGCGCTGGCTCACCCCTACCTGGAGCAGTACTATGACCCGACGGATGAG	46
fas	NM_00004	GGATTGCTCAACAAACCATGCTGGGCATCTTGACCCCTCTACCTCTGGTCTTACGCTCTGTTGCTAGATTATCGTCCAAAGTGTAAATGCC	47

Tables 6A - 6F

Gene	Accession	Name	Sequence	Length	Seq ID.
A-Catenin	NM_001903	S2138/A-Cate.f2	CGTTCCGATCCTCTATACTGCAT	23	94
A-Catenin	NM_001903	S2139/A-Cate.r2	AGGTCCCTGTTGGCCTTATAGG	22	95
A-Catenin	NM_001903	S4725/A-Cate.p2	ATGCCTACAGCACCCCTGATGTCGCA	25	96
AKT1	NM_005163	S0010/AKT1.f3	CGCTTCTATGGCGCTGAGAT	20	97
AKT1	NM_005163	S0012/AKT1.r3	TCCCGGTACACCACGTTCTT	20	98
AKT1	NM_005163	S4776/AKT1.p3	CAGCCCTGGACTACCTGCACTCGG	24	99
AKT2	NM_001626	S0828/AKT2.f3	TCCTGCCACCCTTCAAACC	19	100
AKT2	NM_001626	S0829/AKT2.r3	GGCGGTAAATTCATCATCGAA	21	101
AKT2	NM_001626	S4727/AKT2.p3	CAGGTCACGTCCGAGGTGACACA	24	102
APC	NM_000038	S0022/APC.f4	GGACAGCAGGAATGTGTTTC	20	103
APC	NM_000038	S0024/APC.r4	ACCCACTCGATTTGTTTCTG	20	104
APC	NM_000038	S4888/APC.p4	CATTGGCTCCCCGTGACCTGTA	22	105
B-Catenin	NM_001904	S2150/B-Cate.f3	GGCTCTTGTGCGTACTGTCTT	22	106
B-Catenin	NM_001904	S2151/B-Cate.r3	TCAGATGACGAAGAGCACAGATG	23	107
B-Catenin	NM_001904	S5046/B-Cate.p3	AGGCTCAGTGATGTCTTCCCTGTCACCAG	29	108
Bak	NM_001188	S0037/Bak.f2	CCATTCCCACCATCTACCT	20	109
Bak	NM_001188	S0039/Bak.r2	GGGAACATAGACCCACCAAT	20	110
Bak	NM_001188	S4724/Bak.p2	ACACCCCAGACGTCCTGGCCT	21	111
Bax	NM_004324	S0040/Bax.f1	CCGCCGTGGACACAGACT	18	112
Bax	NM_004324	S0042/Bax.r1	TTGCCGTCAGAAAACATGTCA	21	113
Bax	NM_004324	S4897/Bax.p1	TGCCACTCGGAAAAAGACCTCTCGG	25	114
Bclx	NM_001191	S0046/Bclx.f2	CTTTTGTGGAAGTCTATGGGAACA	24	115
Bclx	NM_001191	S0048/Bclx.r2	CAGCGGTTGAAGCGTTCCT	19	116
Bclx	NM_001191	S4898/Bclx.p2	TTCGGCTCTCGGCTGCTGCA	20	117
BRAF	NM_004333	S3027/BRAF.f2	CCTTCCGACCAGCAGATGAA	20	118
BRAF	NM_004333	S3028/BRAF.r2	TTTATATGCACATTGGGAGCTGAT	24	119
BRAF	NM_004333	S4818/BRAF.p2	CAATTTGGGCAACGAGACCGATCCT	25	120
BRK	NM_005975	S0678/BRK.f2	GTGCAGGAAAGGTTCAAAA	20	121
BRK	NM_005975	S0679/BRK.r2	GCACACACGATGGAGTAAGG	20	122
BRK	NM_005975	S4789/BRK.p2	AGTGTCTGCGTCCAATACACGCGT	24	123
BTC	NM_001729	S1216/BTC.f3	AGGGAGATGCCGCTTCGT	18	124
BTC	NM_001729	S1217/BTC.r3	CTCTCACACCTTGCTCCAATGTA	23	125
BTC	NM_001729	S4844/BTC.p3	CCTTCATCACAGACACAGGAGGGCG	25	126
CA9	NM_001216	S1398/CA9.f3	ATCCTAGCCCTGGTTTTTGG	20	127
CA9	NM_001216	S1399/CA9.r3	CTGCCTTCTCATCTGCACAA	20	128
CA9	NM_001216	S4938/CA9.p3	TTTGCTGTCACCAGCGTCGC	20	129
Cad17	NM_004063	S2186/Cad17.f1	GAAGGCCAAGAACCGAGTCA	20	130
Cad17	NM_004063	S2187/Cad17.r1	TCCCCAGTTAGTTCAAAAAGTCACA	24	131
Cad17	NM_004063	S5038/Cad17.p1	TTATATTCCAGTTTAAGGCCAATCCTC	27	132
CCNA2	NM_001237	S3039/CCNA2.f1	CCATACCTCAAGTATTTGCCATCAG	25	133
CCNA2	NM_001237	S3040/CCNA2.r1	AGCTTTGTCCCGTGACTGTGTA	22	134
CCNA2	NM_001237	S4820/CCNA2.p1	ATTGCTGGAGCTGCCTTTCATTTAGCACT	29	135
CCND3	NM_001760	S2799/CCND3.f1	CCTCTGTGCTACAGATTATACCTTTGC	27	136
CCND3	NM_001760	S2800/CCND3.r1	CACTGCAGCCCCAATGCT	18	137

CCND3	NM_001760	S4966/CCND3.p1	TACCCGCCATCCATGATCGCCA	22	138
CCNE1	NM_001238	S1446/CCNE1.f1	AAAGAAGATGATGACCGGGTTTAC	24	139
CCNE1	NM_001238	S1447/CCNE1.r1	GAGCCTCTGGATGGTGCAAT	20	140
CCNE1	NM_001238	S4944/CCNE1.p1	CAAACCTCAACGTGCAAGCCTCGGA	24	141
CCNE2	NM_057749	S1458/CCNE2.f2	ATGCTGTGGCTCCTTCCTAACT	22	142
CCNE2	NM_057749	S1459/CCNE2.r2	ACCCAAATTGTGATATACAAAAAGGTT	27	143
CCNE2	NM_057749	S4945/CCNE2.p2	TACCAAGCAACCTACATGTCAAGAAAGCCC	30	144
CD105	NM_000118	S1410/CD105.f1	GCAGGTGTCAGCAAGTATGATCAG	24	145
CD105	NM_000118	S1411/CD105.r1	TTTTTCCGCTGTGGTGATGA	20	146
CD105	NM_000118	S4940/CD105.p1	CGACAGGATATTGACCACCGCCTCATT	27	147
CD134	NM_003327	S3138/CD134.f2	GCCCAGTGCGGAGAACAG	18	148
CD134	NM_003327	S3139/CD134.r2	AATCACACGCACCTGGAGAAC	21	149
CD134	NM_003327	S3241/CD134.p2	CCAGCTTGATTCTCGTCTCTGCACTTAAGC	30	150
CD44E	X55150	S3267/CD44E.f1	ATCACCGACAGCACAGACA	19	151
CD44E	X55150	S3268/CD44E.r1	ACCTGTGTTTGGATTTGCAG	20	152
CD44E	X55150	S4767/CD44E.p1	CCCTGCTACCAATATGGACTCCAGTCA	27	153
CD44s	M59040	S3102/CD44s.f1	GACGAAGACAGTCCCTGGAT	20	154
CD44s	M59040	S3103/CD44s.r1	ACTGGGGTGGAATGTGTCTT	20	155
CD44s	M59040	S4826/CD44s.p1	CACCGACAGCACAGACAGAATCCC	24	156
CD44v3	AJ251595v3	S2997/CD44v3.f2	CACACAAAACAGAACCAGGACT	22	157
CD44v3	AJ251595v3	S2998/CD44v3.r2	CTGAAGTAGCACTTCCGGATT	21	157
CD44v3	AJ251595v3	S4814/CD44v3.p2	ACCCAGTGGAACCCAAGCCATTC	23	159
CD44v6	AJ251595v6	S3003/CD44v6.f1	CTCATACCAGCCATCCAATG	20	160
CD44v6	AJ251595v6	S3004/CD44v6.r1	TTGGGTTGAAGAAATCAGTCC	21	161
CD44v6	AJ251595v6	S4815/CD44v6.p1	CACCAAGCCCAGAGGACAGTTCCT	24	162
CD68	NM_001251	S0067/CD68.f2	TGGTTCCAGCCCTGTGT	18	163
CD68	NM_001251	S0069/CD68.r2	CTCCTCCACCCTGGGTTGT	19	164
CD68	NM_001251	S4734/CD68.p2	CTCCAAGCCCAGATTTCAGATTCGAGTCA	28	165
CD82	NM_002231	S0684/CD82.f3	GTGCAGGCTCAGGTGAAGTG	20	166
CD82	NM_002231	S0685/CD82.r3	GACCTCAGGGCGATTTCATGA	20	167
CD82	NM_002231	S4790/CD82.p3	TCAGCTTCTACAACTGGACAGACAACGCTG	30	168
CD9	NM_001769	S0686/CD9.f1	GGGCGTGGAACAGTTTATCT	20	168
CD9	NM_001769	S0687/CD9.r1	CACGGTGAAGGTTTCGAGT	19	170
CD9	NM_001769	S4792/CD9.p1	AGACATCTGCCCAAGAAGGACGT	24	171
CDC25B	NM_021874	S1160/CDC25B.f1	AAACGAGCAGTTTGCCATCAG	21	172
CDC25B	NM_021874	S1161/CDC25B.r1	GTTGGTGATGTTCCGAAGCA	20	176
CDC25B	NM_021874	S4842/CDC25B.p1	CCTCACCGGCATAGACTGGAAGCG	24	174
CEACAM6	NM_002483	S3197/CEACAM.f1	CACAGCCTCACTTCTAACCTTCTG	24	175
CEACAM6	NM_002483	S3198/CEACAM.r1	TTGAATGGCGTGGAATTCAATAG	22	176
CEACAM6	NM_002483	S3261/CEACAM.p1	ACCCACCCACCACTGCCAAGCTC	23	177
CGA	NM_001275	S3221/CGA.f3	CTGAAGGAGCTCCAAGACCT	20	178
CGA	NM_001275	S3222/CGA.r3	CAAAACCGCTGTGTTTCTTC	20	179
CGA	NM_001275	S3254/CGA.p3	TGCTGATGTGCCCTCTCCTTGG	22	180
Chk2	NM_007194	S1434/Chk2.f3	ATGTGGAACCCCCACCTACTT	21	181
Chk2	NM_007194	S1435/Chk2.r3	CAGTCCACAGCACGGTTATACC	22	182
Chk2	NM_007194	S4942/Chk2.p3	AGTCCCAACAGAAACAAGAACTTCAGGCG	29	183
cMet	NM_000245	S0082/cMet.f2	GACATTTCCAGTCCTGCAGTCA	22	184

cMet	NM_000245	S0084/cMet.r2	CTCCGATCGCACACATTTGT	20	185
cMet	NM_000245	S4993/cMet.p2	TGCCTCTCTGCCCCACCCTTTGT	23	186
COX2	NM_000963	S0088/COX2.f1	TCTGCAGAGTTGGAAGCACTCTA	23	187
COX2	NM_000963	S0090/COX2.r1	GCCGAGGCTTTTCTACCAGAA	21	188
COX2	NM_000963	S4995/COX2.p1	CAGGATACAGCTCCACAGCATCGATGTC	28	189
cripto	NM_003212	S3117/cripto.f1	GGGTCTGTGCCCCATGAC	18	190
cripto	NM_003212	S3118/cripto.r1	TGACCGTGCCAGCATTTACA	20	191
cripto	NM_003212	S3237/cripto.p1	CCTGGCTGCCCCAAGAAGTGTTCCCT	25	192
CTSL	NM_001912	S1303/CTSL.f2	GGGAGGCTTATCTCACTGAGTGA	23	193
CTSL	NM_001912	S1304/CTSL.r2	CCATTGCAGCCTTCATTGC	19	194
CTSL	NM_001912	S4899/CTSL.p2	TTGAGGCCAGAGCAGTCTACCAGATTCT	29	195
DCR3	NM_016434	S1786/DCR3.f3	GACCAAGGTCCTGGAATGTC	20	196
DCR3	NM_016434	S1787/DCR3.r3	GTCTTCCCTGTACCCGTAGG	20	197
DCR3	NM_016434	S4982/DCR3.p3	CAGGATGCCATTACCTTCTGCTG	24	198
DIABLO	NM_019887	S0808/DIABLO.f1	CACAATGGCGGCTCTGAAG	19	199
DIABLO	NM_019887	S0809/DIABLO.r1	ACACAAACACTGTCTGTACCTGAAGA	26	200
DIABLO	NM_019887	S4813/DIABLO.p1	AAGTTACGCTGCGCGACAGCCAA	23	201
DPYD	NM_000110	S0100/DPYD.f2	AGGACGCAAGGAGGGTTTG	19	202
DPYD	NM_000110	S0102/DPYD.r2	GATGTCCGCCGAGTCCTTACT	21	203
DPYD	NM_000110	S4998/DPYD.p2	CAGTGCCTACAGTCTCGAGTCTGCCAGTG	29	204
DR5	NM_003842	S2551/DR5.f2	CTCTGAGACAGTGCTTCGATGACT	24	205
DR5	NM_003842	S2552/DR5.r2	CCATGAGGCCCAACTTCCT	19	206
DR5	NM_003842	S4979/DR5.p2	CAGACTTGGTGCCCTTTGACTCC	23	207
EDN1					
endothelin	NM_001955	S0774/EDN1 e.f1	TGCCACCTGGACATCATTTG	20	208
EDN1					
endothelin	NM_001955	S0775/EDN1 e.r1	TGGACCTAGGGCTTCCAAGTC	21	209
EDN1					
endothelin	NM_001955	S4806/EDN1 e.p1	CACTCCCGAGCACGTTGTTCCGT	23	210
EGFR	NM_005228	S0103/EGFR.f2	TGTCGATGGACTTCCAGAAC	20	211
EGFR	NM_005228	S0105/EGFR.r2	ATTGGGACAGCTTGGATCA	19	212
EGFR	NM_005228	S4999/EGFR.p2	CACCTGGGCAGCTGCCAA	18	213
EGFRd27	EGFRd27	S2484/EGFRd2.f2	GAGTCGGGCTCTGGAGGAAAAG	22	214
EGFRd27	EGFRd27	S2485/EGFRd2.r2	CCACAGGCTCGGACGCAC	18	215
EGFRd27	EGFRd27	S4935/EGFRd2.p2	AGCCGTGATCTGTCACCACATAATTACC	28	216
EIF4E	NM_001968	S0106/EIF4E.f1	GATCTAAGATGGCGACTGTCGAA	23	217
EIF4E	NM_001968	S0108/EIF4E.r1	TTAGATTCCGTTTTCTCCTCTTCTG	25	218
EIF4E	NM_001968	S5000/EIF4E.p1	ACCACCCCTACTCCTAATCCCCCGACT	27	219
ErbB3	NM_001982	S0112/ErbB3.f1	CGGTTATGTCATGCCAGATACAC	23	220
ErbB3	NM_001982	S0114/ErbB3.r1	GAAGTGAAGCCCACTGAAGAAAGG	24	221
ErbB3	NM_001982	S5002/ErbB3.p1	CCTCAAAGGTAAGTCCCTCCTCCCGG	25	222
ERBB4	NM_005235	S1231/ERBB4.f3	TGGCTCTTAATCAGTTTCGTTACCT	25	223
ERBB4	NM_005235	S1232/ERBB4.r3	CAAGGCATATCGATCCTCATAAAGT	25	224
ERBB4	NM_005235	S4891/ERBB4.p3	TGTCCACGAATAATGCGTAAATTCTCCAG	30	225
EREG	NM_001432	S0670/EREG.f1	ATAACAAAGTGTAGCTCTGACATGAATG	28	226
EREG	NM_001432	S0671/EREG.r1	CACACCTGCAGTAGTTTTGACTCA	24	227
EREG	NM_001432	S4772/EREG.p1	TTGTTTGCATGGACAGTGCATCTATCTGGT	30	228

ERK1	Z11696	S1560/ERK1.f3	ACGGATCACAGTGGAGGAAG	20	229
ERK1	Z11696	S1561/ERK1.r3	CTCATCCGTCGGGTCATAGT	20	230
ERK1	Z11696	S4882/ERK1.p3	CGCTGGCTCACCCCTACCTG	20	231
fas	NM_000043	S0118/fas.f1	GGATTGCTCAACAACCATGCT	21	232
fas	NM_000043	S0120/fas.r1	GGCATTAACTTTTGGACGATAA	24	233
fas	NM_000043	S5003/fas.p1	TCTGGACCCTCCTACCTCTGGTTCTTACGT	30	234
FRP1	NM_003012	S1804/FRP1.f3	TTGGTACCTGTGGGTTAGCA	20	235
FRP1	NM_003012	S1805/FRP1.r3	CACATCCAAATGCAAACCTGG	20	236
FRP1	NM_003012	S4983/FRP1.p3	TCCCCAGGGTAGAATTCAATCAGAGC	26	237
GPC3	NM_004484	S1835/GPC3.f1	TGATGCGCCTGGAAACAGT	19	238
GPC3	NM_004484	S1836/GPC3.r1	CGAGGTTGTGAAAGGTGCTTATC	23	239
GPC3	NM_004484	S5036/GPC3.p1	AGCAGGCAACTCCGAAGGACAACG	24	240
GRO1	NM_001511	S0133/GRO1.f2	CGAAAAGATGCTGAACAGTGACA	23	241
GRO1	NM_001511	S0135/GRO1.r2	TCAGGAACAGCCACCAGTGA	20	242
GRO1	NM_001511	S5006/GRO1.p2	CTTCCTCCTCCCTTCTGGTCAGTTGGAT	28	243
GUS	NM_000181	S0139/GUS.f1	CCCACTCAGTAGCCAAGTCA	20	244
GUS	NM_000181	S0141/GUS.r1	CACGCAGGTGGTATCAGTCT	20	245
GUS	NM_000181	S4740/GUS.p1	TCAAGTAAACGGGGCTGTTTTCCAAACA	27	246
HB-EGF	NM_001945	S0662/HB-EGF.f1	GACTCCTTCGTCCCCAGTTG	20	247
HB-EGF	NM_001945	S0663/HB-EGF.r1	TGGCACTTGAAGGCTCTGGTA	21	248
HB-EGF	NM_001945	S4787/HB-EGF.p1	TTGGGCCTCCCATAATTGCTTTGCC	25	249
HER2	NM_004448	S0142/HER2.f3	CGGTGTGAGAAGTGCAGCAA	20	250
HER2	NM_004448	S0144/HER2.r3	CCTCTCGCAAGTGCTCCAT	19	251
HER2	NM_004448	S4729/HER2.p3	CCAGACCATAGCACACTCGGGCAC	24	242
HGF	M29145	S1327/HGF.f4	CCGAAATCCAGATGATGATG	20	253
HGF	M29145	S1328/HGF.r4	CCCAAGGAATGAGTGGATTT	20	254
HGF	M29145	S4901/HGF.p4	CTCATGGACCCTGGTGCTACACG	23	255
ID1	NM_002165	S0820/ID1.f1	AGAACCGCAAGGTGAGCAA	19	256
ID1	NM_002165	S0821/ID1.r1	TCCAAGTGAAGGTCCCTGATG	21	257
ID1	NM_002165	S4832/ID1.p1	TGGAGATTCTCCAGCACGTGATCGAC	26	258
IGF1R	NM_000875	S1249/IGF1R.f3	GCATGGTAGCCGAAGATTTCA	21	259
IGF1R	NM_000875	S1250/IGF1R.r3	TTTCCGGTAATAGTCTGTCTCATAGATATC	30	260
IGF1R	NM_000875	S4895/IGF1R.p3	CGCGTCATACCAAAATCTCCGATTTTGA	28	261
IGFBP3	NM_000598	S0157/IGFBP3.f3	ACGCACCGGGTGTCTGA	17	262
IGFBP3	NM_000598	S0159/IGFBP3.r3	TGCCCTTTCTTGATGATGATTATC	24	263
IGFBP3	NM_000598	S5011/IGFBP3.p3	CCCAAGTTCCACCCCTCCATTCA	24	264
IRS1	NM_005544	S1943/IRS1.f3	CCACAGCTCAGCTTCTGTCA	20	265
IRS1	NM_005544	S1944/IRS1.r3	CCTCAGTGCCAGTCTCTTCC	20	266
IRS1	NM_005544	S5050/IRS1.p3	TCCATCCCAGCTCCAGCCAG	20	267
ITGA3	NM_002204	S2347/ITGA3.f2	CCATGATCCTCACTCTGCTG	20	268
ITGA3	NM_002204	S2348/ITGA3.r2	GAAGCTTTGTAGCCGGTGAT	20	269
ITGA3	NM_002204	S4852/ITGA3.p2	CACTCCAGACCTCGCTTAGCATGG	24	270
ITGB3	NM_000212	S3126/ITGB3.f1	ACCGGGAGCCCTACATGAC	19	271
ITGB3	NM_000212	S3127/ITGB3.r1	CCTTAAGCTCTTCACTGACTCAATCT	27	272
ITGB3	NM_000212	S3243/ITGB3.p1	AAATACCTGCAACCGTTACTGCCGTGAC	28	273
KRT17	NM_000422	S0172/KRT17.f2	CGAGGATTGGTTCTTCAGCAA	21	274
KRT17	NM_000422	S0174/KRT17.r2	ACTCTGCACCAGCTCACTGTTG	22	275

KRT17	NM_000422	S5013/KRT17.p2	CACCTCGCGGTTCAAGTTCCTCTGT	24	276
LAMC2	NM_005562	S2826/LAMC2.f2	ACTCAAGCGGAAATTGAAGCA	21	277
LAMC2	NM_005562	S2827/LAMC2.r2	ACTCCCTGAAGCCGAGACACT	21	278
LAMC2	NM_005562	S4969/LAMC2.p2	AGGTCTTATCAGCACAGTCTCCGCCTCC	28	278
MTA1	NM_004689	S2369/MTA1.f1	CCGCCCTCACCTGAAGAGA	19	280
MTA1	NM_004689	S2370/MTA1.r1	GGAATAAGTTAGCCGCGCTTCT	22	281
MTA1	NM_004689	S4855/MTA1.p1	CCCAGTGTCCGCCAAGGAGCG	21	282
NMYC	NM_005378	S2884/NMYC.f2	TGAGCGTCGCAGAAACCA	18	283
NMYC	NM_005378	S2885/NMYC.r2	TCCCTGAGCGTGAGAAAGCT	20	284
NMYC	NM_005378	S4976/NMYC.p2	CCAGCGCCGCAACGACCTTC	20	285
p14ARF	NM_000077	S0199/p14ARF.f3	GCGGAAGGTCCCTCAGACA	19	286
p14ARF	NM_000077	S0201/p14ARF.r3	TCTAAGTTTCCCGAGGTTTCTCA	23	297
p14ARF	NM_000077	S5068/p14ARF.p3	CCCCGATTGAAAGAACCAGAGAGGCT	26	288
p27	NM_004064	S0205/p27.f3	CGGTGGACCACGAAGAGTTAA	21	289
p27	NM_004064	S0207/p27.r3	GGCTCGCCTCTTCCATGTC	19	290
p27	NM_004064	S4750/p27.p3	CCGGGACTTGAGAAAGCACTGCA	23	291
P53	NM_000546	S0208/P53.f2	CTTGAACCCTTGCTTGCAA	20	292
P53	NM_000546	S0210/P53.r2	CCCGGGACAAAGCAAATG	18	293
P53	NM_000546	S5065/P53.p2	AAGTCCTGGGTGCTTCTGACGCACA	25	294
PAI1	NM_000602	S0211/PAI1.f3	CCGCAACGTGGTTTTCTCA	19	295
PAI1	NM_000602	S0213/PAI1.r3	TGCTGGGTTTCTCCTCCTGTT	21	296
PAI1	NM_000602	S5066/PAI1.p3	CTCGGTGTTGGCCATGCTCCAG	22	297
PDGFA	NM_002607	S0214/PDGFA.f3	TTGTTGGTGTGCCCTGGTG	19	298
PDGFA	NM_002607	S0216/PDGFA.r3	TGGGTTCTGTCCAAACACTGG	21	299
PDGFA	NM_002607	S5067/PDGFA.p3	TGGTGGCGGTCACTCCCTCTGC	22	300
PDGFB	NM_002608	S0217/PDGFB.f3	ACTGAAGGAGACCCTTGAG	20	301
PDGFB	NM_002608	S0219/PDGFB.r3	TAAATAACCCTGCCACACA	20	302
PDGFB	NM_002608	S5014/PDGFB.p3	TCTCCTGCCGATGCCCTAGG	21	303
PGK1	NM_000291	S0232/PGK1.f1	AGAGCCAGTTGCTGTAGAACTCAA	24	304
PGK1	NM_000291	S0234/PGK1.r1	CTGGGCCTACACAGTCCTTCA	21	305
PGK1	NM_000291	S5022/PGK1.p1	TCTCTGCTGGGCAAGGATGTTCTGTTC	27	306
PLAUR	NM_002659	S1976/PLAUR.f3	CCCATGGATGCTCCTCTGAA	20	307
PLAUR	NM_002659	S1977/PLAUR.r3	CCGGTGGCTACCAGACATTG	20	308
PLAUR	NM_002659	S5054/PLAUR.p3	CATTGACTGCCGAGGCCCATG	22	309
PPARG	NM_005037	S3090/PPARG.f3	TGACTTTATGGAGCCCAAGTT	21	310
PPARG	NM_005037	S3091/PPARG.r3	GCCAAGTCGCTGTCATCTAA	20	311
PPARG	NM_005037	S4824/PPARG.p3	TTCCAGTGCATTGAACTTCACAGCA	25	312
PTPD1	NM_007039	S3069/PTPD1.f2	CGCTTGCCCTAACTCATACTTCC	23	313
PTPD1	NM_007039	S3070/PTPD1.r2	CCATTCAGACTGCGCCACTT	20	314
PTPD1	NM_007039	S4822/PTPD1.p2	TCCACGCAGCGTGGCACTG	19	315
RANBP2	NM_006267	S3081/RANBP2.f3	TCCTTCAGCTTTCACACTGG	20	316
RANBP2	NM_006267	S3082/RANBP2.r3	AAATCCTGTTCCACCTGAC	20	317
RANBP2	NM_006267	S4823/RANBP2.p3	TCCAGAAGAGTCATGCAACTTCATTTCTG	29	318
RASSF1	NM_007182	S2393/RASSF1.f3	AGTGGGAGACACCTGACCTT	20	319
RASSF1	NM_007182	S2394/RASSF1.r3	TGATCTGGGCATTGTACTCC	20	320
RASSF1	NM_007182	S4909/RASSF1.p3	TTGATCTTCTGCTCAATCTCAGCTTGAGA	29	321
RB1	NM_000321	S2700/RB1.f1	CGAAGCCCTTACAAGTTTCC	20	322

RB1	NM_000321	S2701/RB1.r1	GGACTCTTCAGGGGTGAAAT	20	323
RB1	NM_000321	S4765/RB1.p1	CCCTTACGGATTCTGGAGGGAAC	24	324
RIZ1	NM_012231	S1320/RIZ1.f2	CCAGACGAGCGATTAGAAGC	20	325
RIZ1	NM_012231	S1321/RIZ1.r2	TCCTCCTCTTCCTCCTCCTC	20	326
RIZ1	NM_012231	S4761/RIZ1.p2	TGTGAGGTGAATGATTTGGGGGA	23	327
RPLPO	NM_001002	S0256/RPLPO.f2	CCATTCTATCATCAACGGGTACAA	24	328
RPLPO	NM_001002	S0258/RPLPO.r2	TCAGCAAGTGGGAAGGTGTAATC	23	329
RPLPO	NM_001002	S4744/RPLPO.p2	TCTCCACAGACAAGGCCAGGACTCG	25	330
SPRY2	NM_005842	S2985/SPRY2.f2	TGTGGCAAGTGCAAATGTAA	20	331
SPRY2	NM_005842	S2986/SPRY2.r2	GTCGCAGATCCAGTCTGATG	20	332
SPRY2	NM_005842	S4811/SPRY2.p2	CAGAGGCCTTGGGTAGGTGCACTC	24	333
Src	NM_004383	S1820/Src.f2	CCTGAACATGAAGGAGCTGA	20	334
Src	NM_004383	S1821/Src.r2	CATCACGTCTCCGAACCTCC	19	335
Src	NM_004383	S5034/Src.p2	TCCCGATGGTCTGCAGCAGCT	21	336
STK15	NM_003600	S0794/STK15.f2	CATCTTCCAGGAGGACCACT	20	337
STK15	NM_003600	S0795/STK15.r2	TCCGACCTTCAATCATTTCA	20	338
STK15	NM_003600	S4745/STK15.p2	CTCTGTGGCACCCCTGGACTACCTG	24	339
SURV	NM_001168	S0259/SURV.f2	TGTTTTGATTCCCGGGCTTA	20	340
SURV	NM_001168	S0261/SURV.r2	CAAAGCTGTCAGCTCTAGCAAAAG	24	341
SURV	NM_001168	S4747/SURV.p2	TGCCTTCTCCTCCCTCACTTCTCACCT	28	342
TERC	U86046	S2709/TERC.f2	AAGAGGAACGGAGCGAGTC	19	343
TERC	U86046	S2710/TERC.r2	ATGTGTGAGCCGAGTCCTG	19	344
TERC	U86046	S4958/TERC.p2	CACGTCCCACAGCTCAGGGAATC	23	345
TFRC	NM_003234	S1352/TFRC.f3	GCCAACTGCTTTTCAATTTGTG	20	346
TFRC	NM_003234	S1353/TFRC.r3	ACTCAGGCCCATTTCTTTA	20	347
TFRC	NM_003234	S4748/TFRC.p3	AGGGATCTGAACCAATACAGAGCAGACA	28	348
TGFBR2	NM_003242	S2422/TGFBR2.f3	AACACCAATGGGTTCCATCT	20	349
TGFBR2	NM_003242	S2423/TGFBR2.r3	CCTCTTCATCAGGCCAAACT	20	350
TGFBR2	NM_003242	S4913/TGFBR2.p3	TTCTGGGCTCCTGATTGCTCAAGC	24	351
TIMP2	NM_003255	S1680/TIMP2.f1	TCACCCTCTGTGACTTCATCGT	22	352
TIMP2	NM_003255	S1681/TIMP2.r1	TGTGGTTCAGGCTCTTCTTCTG	22	353
TIMP2	NM_003255	S4916/TIMP2.p1	CCCTGGGACACCCTGAGCACCA	22	354
TITF1	NM_003317	S2224/TITF1.f1	CGACTCCGTTCTCAGTGTCTGA	22	355
TITF1	NM_003317	S2225/TITF1.r1	CCCTCCATGCCCACTTTCT	19	356
TITF1	NM_003317	S4829/TITF1.p1	ATCTTGAGTCCCCTGGAGGAAAGC	24	357
TP53BP1	NM_005657	S1747/TP53BP.f2	TGCTGTTGCTGAGTCTGTTG	20	358
TP53BP1	NM_005657	S1748/TP53BP.r2	CTTGCCCTGGCTTCACAGATA	20	359
TP53BP1	NM_005657	S4924/TP53BP.p2	CCAGTCCCCAGAAGACCATGTCTG	24	360
upa	NM_002658	S0283/upa.f3	GTGGATGTGCCCTGAAGGA	19	361
upa	NM_002658	S0285/upa.r3	CTGCGGATCCAGGGTAAGAA	20	362
upa	NM_002658	S4769/upa.p3	AAGCCAGGCGTCTACACGAGAGTCTCAC	28	363
VEGFC	NM_005429	S2251/VEGFC.f1	CCTCAGCAAGACGTTATTTGAAATT	25	364
VEGFC	NM_005429	S2252/VEGFC.r1	AAGTGTGATTGGCAAAACTGATTG	24	365
VEGFC	NM_005429	S4758/VEGFC.p1	CCTCTCTCTCAAGGCCCCAAACCAGT	26	366
XIAP	NM_001167	S0289/XIAP.f1	GCAGTTGGAAGACACAGGAAAGT	23	367
XIAP	NM_001167	S0291/XIAP.r1	TGCGTGGCACTATTTTCAAGA	21	368
XIAP	NM_001167	S4752/XIAP.p1	TCCCCAAATTGCAGATTTATCAACGGC	27	369

YB-1	NM_004559	S1194/YB-1.f2	AGACTGTGGAGTTTGATGTTGTTGA	25	370
YB-1	NM_004559	S1195/YB-1.r2	GGAACACCACCAGGACCTGTAA	22	371
YB-1	NM_004559	S4843/YB-1.p2	TTGCTGCCTCCGCACCCTTTTCT	23	372